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

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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 (2–8). 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 responses.

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

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[2] The abbreviations used are: BCR, B cell receptor; MVB, multivesicular body; MIIC, MHC class II complex; ITAM, immunoreceptor tyrosine-based activation motif; Syk, spleen tyrosine kinase; Cbl, Casitas B-lineage lymphoma; PC, phosphorylcholine; HEL, hen egg lysozyme; DMSO, dimethyl sulfoxide; F-P, fluid-phase; SA, streptavidin; MFI, mean fluorescent intensity.

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^b BCR and $I\text{-}A^k$ class II) and MD4 B10.Br (expressing a HEL-specific IgM^a BCR and $I\text{-}A^k$ 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, L-glutamine, 500 μg/ml penicillin-streptomycin and 50 μM 2-mercaptoethanol. Cells were maintained at a density of $< 1 \times 10^6$ viable cells/ml at 37 °C, 5% CO_2.

Reagents—The following reagents were used for this study: hen egg lysozyme (HEL, catalog no. L-6876, Sigma), HEL_{46-61}-$I\text{-}A^k$-specific monoclonal antibody C4H3 (26), goat anti-mouse IgG $F(ab')_2$ (catalog no. 115-006-006, Jackson ImmunoResearch, West Grove, PA), FITC rat anti-mouse IgG_1 (A85-1, catalog no. 553443, BD Biosciences), FITC anti-mouse $I\text{-}A^k$ (10-3.6, IgG_{2a}, catalog no. 55352, BD Biosciences), FITC anti-mouse IgM^a (DS-1, IgG_1, catalog no. 553516, BD Biosciences), PE anti-CD45R/B220 (RA3-6B2, IgG_{2a}, 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^a (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 HEL_{46-61}-$I\text{-}A^k$ 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; 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; 4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo [3,4-d]-pyrimidine + 4-amino-5-(4-chlorophenyl)-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-$muIgM^b$ 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 HEL_{46-61}-$I\text{-}A^k$ expressed at 24 h was determined by staining with HEL_{46-61}-$I\text{-}A^k$ -specific mAb C4H3 followed by anti-rat IgG_{2b}-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 $I\text{-}A^k$ expression in parallel samples.

c-Cbl and Cbl-b Knockdown in A20uWT Cells—High-titer ($1.8 - 4.72 \times 10^8$ TU/ml) viral particles containing Expression Arrest™ GIPZ lentiviral shRNAmir 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, AK045005, BC035536, and AK084162), GAPDH (positive control, pool of six, catalog no. RHS4372, GenBank™ accession no. NM_008084.2), 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. 3*A*. 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 2747, 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-huIgM-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 coverslips, 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 $p \leq 0.05$.

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. 1*A*) or splenic B cells (supplemental Fig. S1*A*) 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. 1*B*) (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. 1*D*). Similar results were obtained in splenic B cells (supplemental Fig. S1*C*). 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. 2*A*, 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. 2*B*) 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. 3*A*). Nevertheless, they

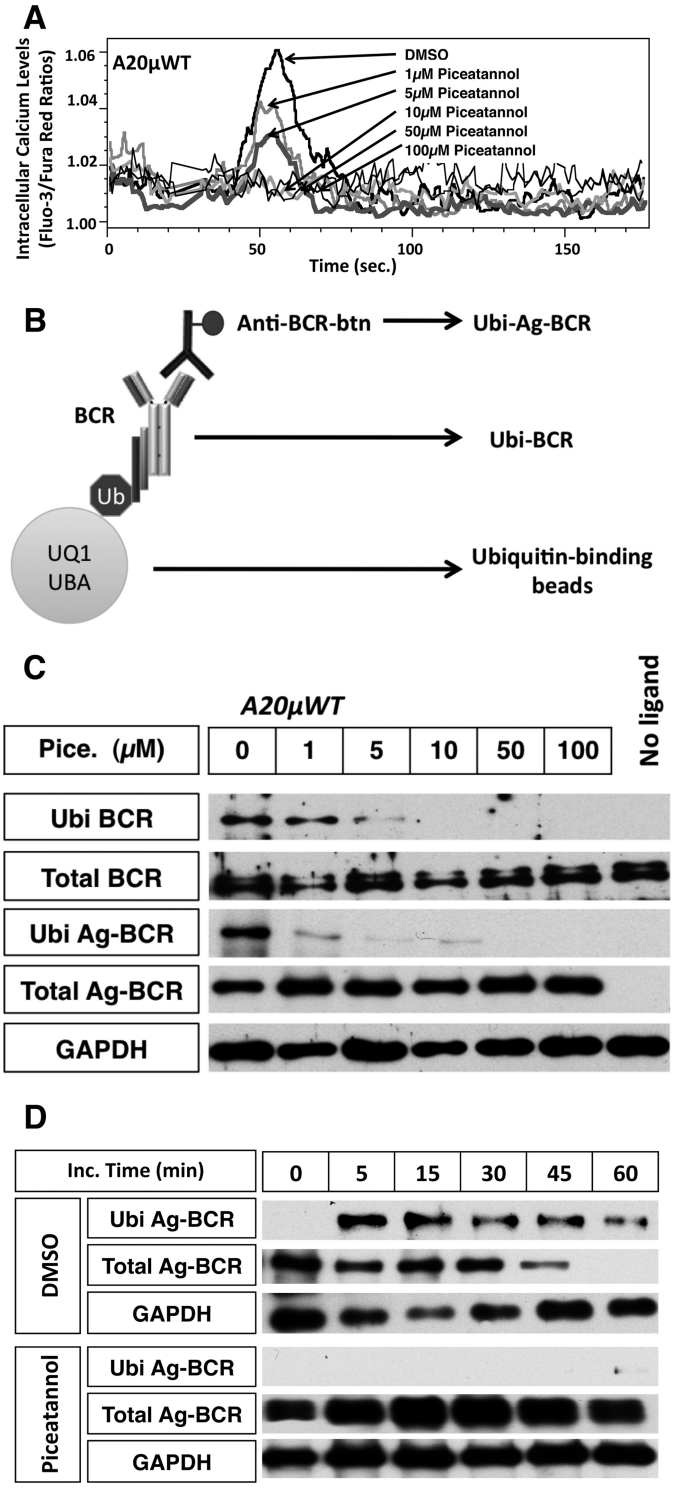

FIGURE 1. **Inhibition of Syk activity abrogates BCR ubiquitination.** *A*, fluo-3- and Fura Red-loaded A20μWT B cells were pretreated with the indicated dose of piceatannol or diluted vehicle (DMSO), stimulated with anti-human IgM antibody, and then the resulting intracellular calcium flux was determined by flow cytometry. Shown are representative traces from one of three independent experiments. *B*, graphical representation of the ubiquitin (*Ubi*) pull-down approach. *C*, A20μWT B cells were pretreated for 1 h at 37 °C with indicated concentrations of piceatannol (*Pice*) or diluted vehicle (DMSO) and then pulsed with anti-human IgM-btn (anti-BCR) for the 20 min. Cells were then lysed, and a fraction of whole cell lysate was retained. Ubiquitinated ligand-BCR complexes were isolated from the remaining whole cell lysate by UQ1 pull-down. BCR (*Total BCR* from the whole cell lysate and ubiquitinated BCR and *Ubi-BCR* from the UQ1 pull-down) was detected by SDS-PAGE and anti-IgM Western blot analysis. Ligand-BCR (*Total Ag·BCR* from the whole cell lysate and ubiquitinated Ag·BCR and *Ubi-Ag·BCR* from the UQ1 pull-down) was detected by SDS-PAGE and SA-HRP Western blot analysis. GAPDH serves as a loading control for total BCR/Ag·BCR. Shown are representative results from one of three independent experiments. *D*, A20μWT B cells were pretreated for 1 h at 37 °C with 10 μM piceatannol or diluted vehicle (DMSO) and then pulsed with anti-human IgM-btn for the indicated times. Cells were then lysed, and total Ag·BCR and ubiquitinated ligand-BCR complexes were detected by SDS-PAGE and blotting with SA-HRP as in *C*. GAPDH was used as a loading control. Shown are representative results from one of three independent experiments.

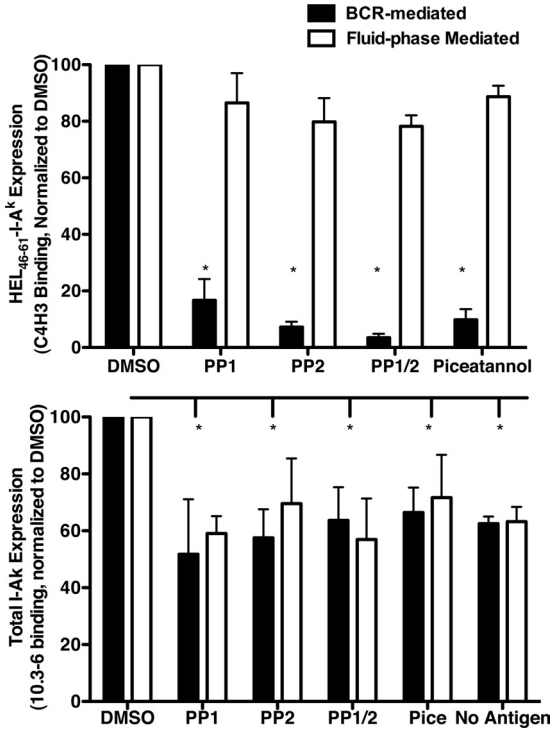

FIGURE 2. **BCR-mediated antigen presentation requires Src-family kinase and Syk signaling.** *A*, splenocytes from MD4.B10.Br (*black bars*, BCR-mediated) or B10.Br (*white bars*, fluid-phase mediated) mice were preincubated for 1 h with indicated inhibitors or diluted vehicle (DMSO). Cells were then 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_{46-61}-I-A^k complex expression was determined by staining with the HEL_{46-61}-I-A^k complex-specific mAb C4H3 and analysis flow cytometry. Shown is the normalized MFI of C4H3 staining of B220+ 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^k monoclonal antibody (10–3.6-FITC) and analyzed by flow cytometry. Shown is the normalized MFI of 10–3.6 staining of B220+ 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. *, $p \leq 0.05$. *Pice*, piceatannol.

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.

A20μWT B cells express both c-Cbl and Cbl-b (Fig. 3*B*). To selectively knock down the levels of either c-Cbl or Cbl-b, A20μWT 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. 3*A*). After 7 days of post-infection growth

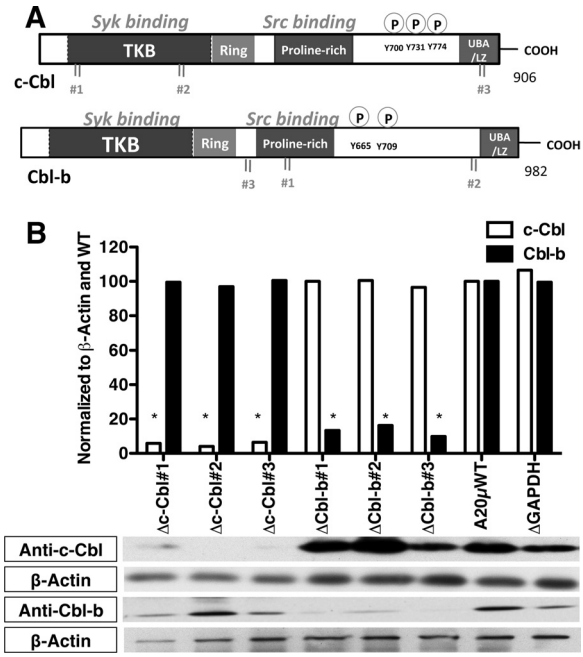

FIGURE 3. **Stable knockdown of the ubiquitin E3 ligases c-Cbl and Cbl-b in A20μWT B cells.** *A*, domain organization and comparison of c-Cbl and Cbl-b E3 ubiquitin ligases. There is 50% similarity between the two proteins at the amino acid level and 55% similarity at the nucleotide level, with most of the differences between the two proteins restricted to the C-terminal ends of the proteins. The tyrosine kinase binding (*TKB*) domain is responsible for binding the Tyr-323 phosphorylated form of Syk. *#1*, *#2*, *#3* indicate the shRNA sense binding sites. *B*, SDS-PAGE and anti-Cbl Western blot analysis of whole cell lysates. Densitometry results (derived from Western blot analyses from three independent experiments) were normalized first to the loading control β-actin and then to the level of Cbl in WT control cells. Student's *t* test was used to compare the level of Cbl in knockdown *versus* WT cells. *, $p \leq 0.05$.

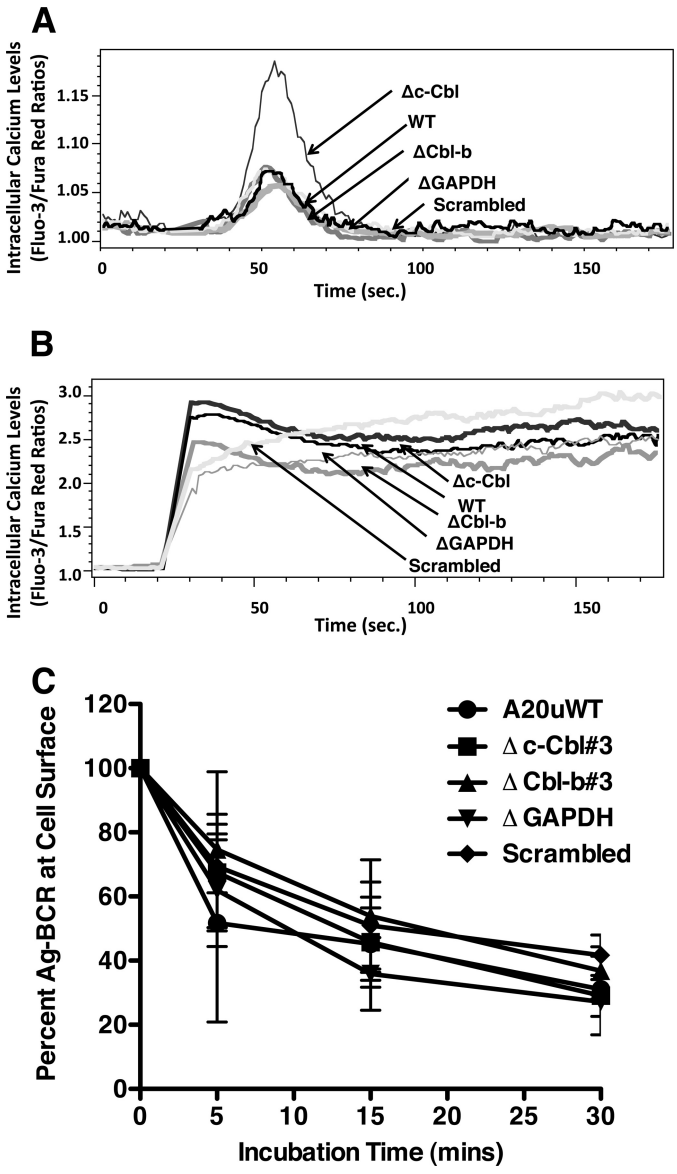

FIGURE 4. **The effect of selective Cbl knockdown on BCR signaling and endocytosis.** *A* and *B*, fluo-3- and Fura Red-loaded B cells were stimulated with anti-human IgM antibody (*A*) or calcium ionophore (*B*), and the resulting intracellular calcium flux was monitored by flow cytometry. BCR-elicited calcium signaling is selectively increased in Δc-Cbl B cells. Shown are representative tracings from one of three independent experiments. *C*, biotinylated anti-huIgM was bound to B cells on ice. The cells were washed, incubated at 37 °C for the indicated time, and then the fraction of anti-BCR-btn left at the surface was determined by SA-Alexa Fluor 488 staining and analysis by flow cytometry. Shown is the average level of surface ligand at each time point from three independent experiments ($p = 0.75$).

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*B*). 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 Δc-Cbl and ΔCbl-b from here on) were selected to be the focus of further analysis. A20μWT cells expressing either GAPDH and scrambled (non-silencing) shRNA (as well as non-infected A20μWT 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 A20μWT cells ± ~10%. Knockdown of c-Cbl, an established negative regulator of Syk activity (23, 34), results in an increase in BCR-driven calcium signaling (Fig. 4*A* and supplemental Fig. S2*A*). In contrast, knockdown of Cbl-b has no detectable impact on this early aspect of BCR signaling (Fig. 4*A* and supplemental Fig. S2*A*). 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. 4*C* and supplemental Fig. S2*B*). 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 is 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

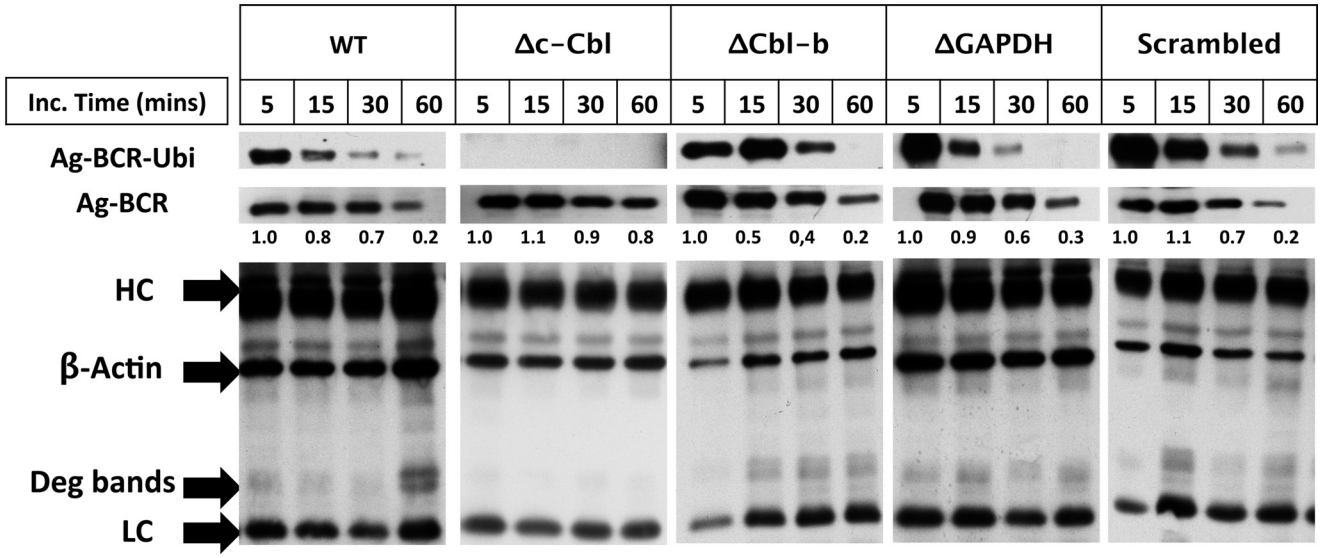

FIGURE 5. **c-Cbl mediates Ag·BCR ubiquitination and processing.** *Upper panel*, B cells were pulsed with anti-human IgM-btn for the indicated time (minutes) at 37 °C. The cells were lysed and ubiquitinated ligand-BCR complexes isolated by ubiquitin pull-down of a fraction of the whole cell lysate (Fig. 1*C*). The level of total ligand-BCR complexes (*Ag·BCR*) and ubiquitinated ligand-BCR complexes (*Ag·BCR-Ubi*) was determined by SDS-PAGE and SA-HRP blotting of whole cell lysate and ubiquitin pull-down, respectively. The numbers below the blots represent the normalized level of total IgM heavy chain detected in each sample (average from three independent experiments). *Lower panel*, longer exposure of SA-HRP blots of whole cell lysate (also probed with anti-β-actin antibody). *HC* and *LC*, intact heavy and light chains of anti-BCR-btn antibody; *Deg bands*, degradation bands of anti-BCR-btn HC).

molecules with biotinylated anti-BCR antibodies and our established ubiquitin pull-down assay (Fig. 1*B*). 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$^+$ late endosomes and lysosomes within 30 min (Fig. 6 and supplemental Fig. S3), a time that is coincident with or slightly preceding the detection of ligand-BCR degradation products (Fig. 5). In contrast, internalized ligand-BCR complexes within Δc-Cbl cells were not found to extensively colocalize with 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 A20μWT 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

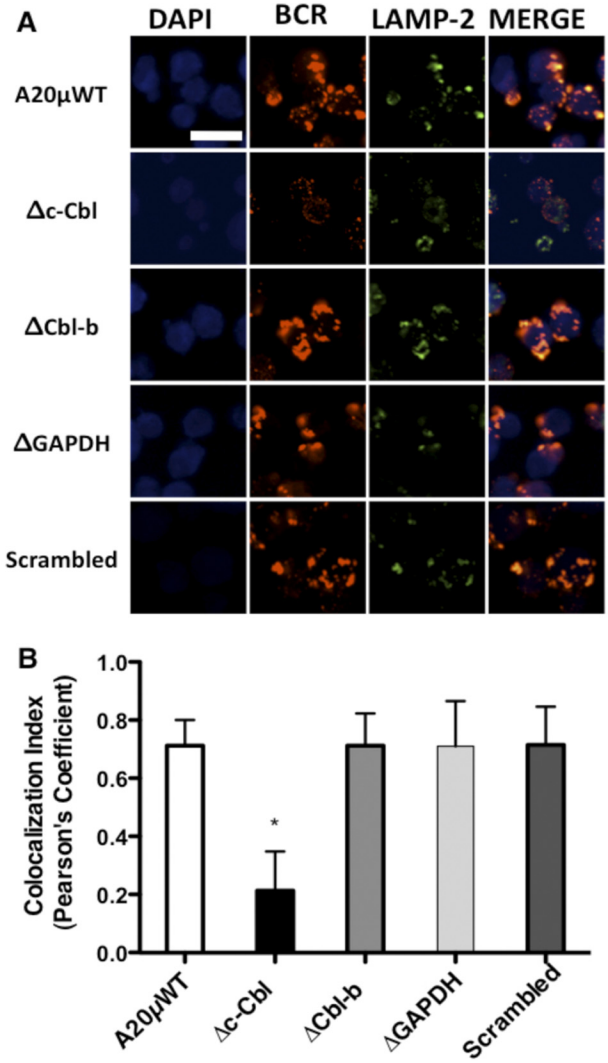

FIGURE 6. **c-Cbl mediates Ag·BCR trafficking to LAMP-2$^+$ endocytic compartments.** *A*, B cells were pulsed on ice with 10 μg/ml anti-hulgM-btn followed by SA-Alexa Fluor 594, washed, and then incubated at 37 °C for 30 min. Cells were then fixed, permeabilized, and stained with anti-CD107b (LAMP-2). 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. *, $p \leq 0.05$.

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 processing/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 effects 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 the 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

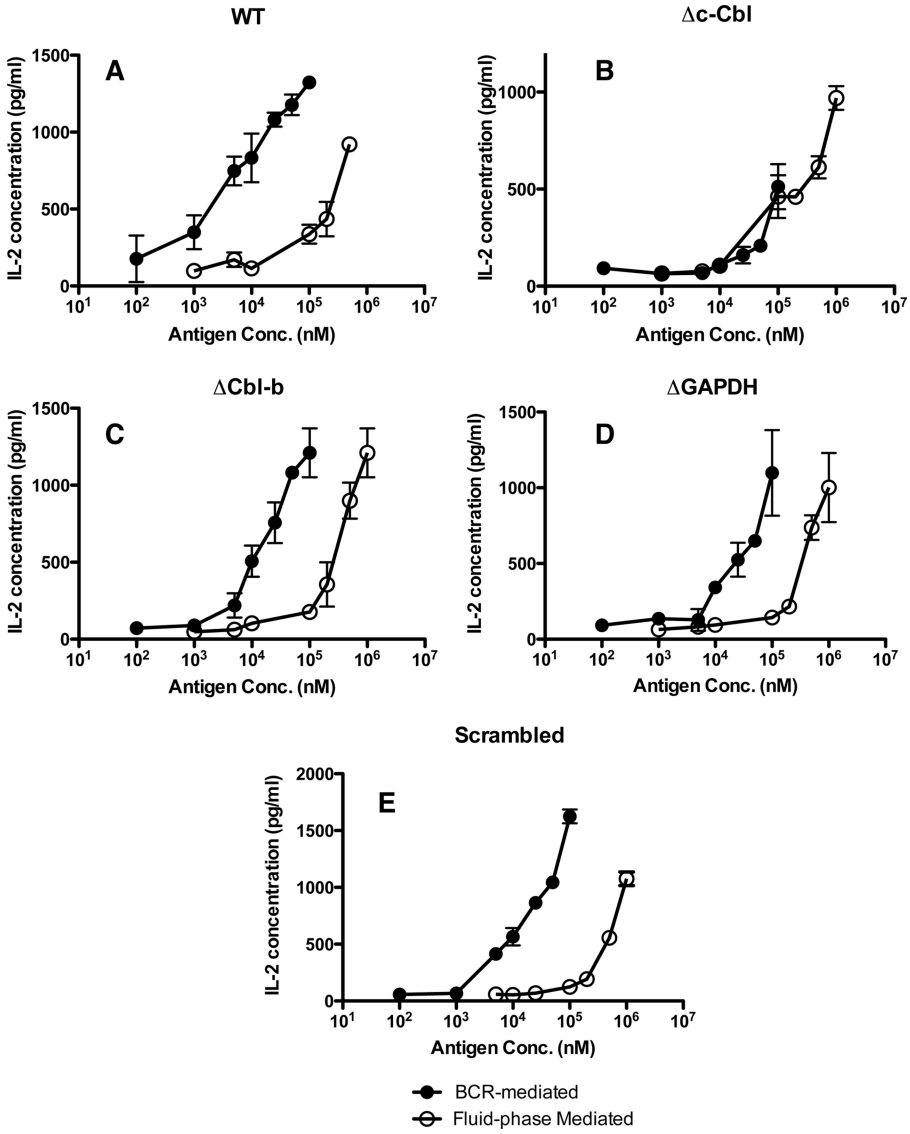

FIGURE 7. **c-Cbl is essential for BCR-mediated antigen presentation.** The indicated B cells (all expressing a PC-specific BCR) were cocultured with DO-11.10 T cells and the indicated dose of either PC-Ova (for BCR-mediated antigen processing) or Ova (for fluid-phase antigen processing). An ELISA was used to determine the level of DO-11.10 produced IL-2 in the culture supernatants. Shown are representative results from one of three independent experiments. Values are an average of three measurements. Presentation of PC-Ova is significantly different ($p < 0.01$) from that of Ova in all cells except Δc-Cbl B cells.

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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REFERENCES

1. Trombetta, E. S., and Mellman, I. (2005) Cell biology of antigen processing *in vitro* and *in vivo*. *Annu. Rev. Immunol.* **23,** 975–1028
2. Hombach, J., Leclercq, L., Radbruch, A., Rajewsky, K., and Reth, M. (1988) A novel 34-kd protein co-isolated with the IgM molecule in surface IgM-expressing cells. *EMBO J.* **7,** 3451–3456
3. Hombach, J., Tsubata, T., Leclercq, L., Stappert, H., and Reth, M. (1990) Molecular components of the B-cell antigen receptor complex of the IgM class. *Nature* **343,** 760–762
4. Bonnerot, C., Lankar, D., Hanau, D., Spehner, D., Davoust, J., Salamero, J., and Fridman, W. H. (1995) Role of B cell receptor Ig α and Ig β subunits in MHC class II-restricted antigen presentation. *Immunity* **3,** 335–347
5. Lankar, D., Briken, V., Adler, K., Weiser, P., Cassard, S., Blank, U., Viguier, M., and Bonnerot, C. (1998) Syk tyrosine kinase and B cell antigen receptor (BCR) immunoglobulin-α subunit determine BCR-mediated major histocompatibility complex class II-restricted antigen presentation. *J. Exp. Med.* **188,** 819–831
6. Lankar, D., Vincent-Schneider, H., Briken, V., Yokozeki, T., Raposo, G., and Bonnerot, C. (2002) Dynamics of major histocompatibility complex

class II compartments during B cell receptor-mediated cell activation. *J. Exp. Med.* **195,** 461–472
7. Reth, M., Hombach, J., Wienands, J., Campbell, K. S., Chien, N., Justement, L. B., and Cambier, J. C. (1991) The B-cell antigen receptor complex. *Immunol. Today* **12,** 196–201
8. Reth, M., Wienands, J., Tsubata, T., and Hombach, J. (1991) Identification of components of the B cell antigen receptor complex. *Adv. Exp. Med. Biol.* **292,** 207–214
9. Mitchell, R. N., Barnes, K. A., Grupp, S. A., Sanchez, M., Misulovin, Z., Nussenzweig, M. C., and Abbas, A. K. (1995) Intracellular targeting of antigens internalized by membrane immunoglobulin in B lymphocytes. *J. Exp. Med.* **181,** 1705–1714
10. Barnes, K. A., and Mitchell, R. N. (1995) Detection of functional class II-associated antigen. Role of a low density endosomal compartment in antigen processing. *J. Exp. Med.* **181,** 1715–1727
11. Drake, J. R., Lewis, T. A., Condon, K. B., Mitchell, R. N., and Webster, P. (1999) Involvement of MIIC-like late endosomes in B cell receptor-mediated antigen processing in murine B cells. *J. Immunol.* **162,** 1150–1155
12. Drake, J. R., Webster, P., Cambier, J. C., and Mellman, I. (1997) Delivery of B cell receptor-internalized antigen to endosomes and class II vesicles. *J. Exp. Med.* **186,** 1299–1306
13. Song, W., Cho, H., Cheng, P., and Pierce, S. K. (1995) Entry of B cell antigen receptor and antigen into class II peptide-loading compartment is independent of receptor cross-linking. *J. Immunol.* **155,** 4255–4263
14. Kleijmeer, M. J., Raposo, G., and Geuze, H. J. (1996) Characterization of MHC class II compartments by immunoelectron microscopy *Methods* **10,** 191–207
15. Hicke, L. (2001) A new ticket for entry into budding vesicles. Ubiquitin. *Cell* **106,** 527–530
16. Drake, L., McGovern-Brindisi, E. M., and Drake, J. R. (2006) BCR ubiquitination controls BCR-mediated antigen processing and presentation. *Blood* **108,** 4086–4093
17. Zhang, M., Veselits, M., O'Neill, S., Hou, P., Reddi, A. L., Berlin, I., Ikeda, M., Nash, P. D., Longnecker, R., Band, H., and Clark, M. R. (2007) Ubiquitinylation of Ig β dictates the endocytic fate of the B cell antigen receptor. *J. Immunol.* **179,** 4435–4443
18. Katkere, B., Rosa, S., Caballero, A., Repasky, E. A., and Drake, J. R. (2010) Physiological-range temperature changes modulate cognate antigen processing and presentation mediated by lipid raft-restricted ubiquitinated B cell receptor molecules. *J. Immunol.* **185,** 5032–5039
19. DeFranco, A. L., Richards, J. D., Blum, J. H., Stevens, T. L., Law, D. A., Chan, V. W., Datta, S. K., Foy, S. P., Hourihane, S. L., and Gold, M. R. (1995) Signal transduction by the B-cell antigen receptor. *Ann. N.Y. Acad. Sci.* **766,** 195–201
20. Le Roux, D., Lankar, D., Yuseff, M. I., Vascotto, F., Yokozeki, T., Faure-André, G., Mougneau, E., Glaichenhaus, N., Manoury, B., Bonnerot, C., and Lennon-Duménil, A. M. (2007) Syk-dependent actin dynamics regulate endocytic trafficking and processing of antigens internalized through the B-cell receptor. *Mol. Biol. Cell* **18,** 3451–3462
21. Yasuda, T., Maeda, A., Kurosaki, M., Tezuka, T., Hironaka, K., Yamamoto, T., and Kurosaki, T. (2000) Cbl suppresses B cell receptor-mediated phospholipase C (PLC)-γ2 activation by regulating B cell linker protein-PLC-γ2 binding. *J. Exp. Med.* **191,** 641–650
22. Yasuda, T., Tezuka, T., Maeda, A., Inazu, T., Yamanashi, Y., Gu, H., Kurosaki, T., and Yamamoto, T. (2002) Cbl-b positively regulates Btk-mediated activation of phospholipase C-γ2 in B cells. *J. Exp. Med.* **196,** 51–63
23. Kitaura, Y., Jang, I. K., Wang, Y., Han, Y. C., Inazu, T., Cadera, E. J., Schlissel, M., Hardy, R. R., and Gu, H. (2007) Control of the B cell-intrinsic tolerance programs by ubiquitin ligases Cbl and Cbl-b. *Immunity* **26,** 567–578
24. Rao, N., Dodge, I., and Band, H. (2002) The Cbl family of ubiquitin ligases. Critical negative regulators of tyrosine kinase signaling in the immune system. *J. Leukoc. Biol.* **71,** 753–763
25. Putnam, M. A., Moquin, A. E., Merrihew, M., Outcalt, C., Sorge, E., Caballero, A., Gondré-Lewis, T. A., and Drake, J. R. (2003) Lipid raft-independent B cell receptor-mediated antigen internalization and intracellular trafficking. *J. Immunol.* **170,** 905–912
26. Zhong, G., Reis e Sousa, C., and Germain, R. N. (1997) Production, specificity, and functionality of monoclonal antibodies to specific peptide-major histocompatibility complex class II complexes formed by processing of exogenous protein. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 13856–13861
27. Caballero, A., Katkere, B., Wen, X. Y., Drake, L., Nashar, T. O., and Drake, J. R. (2006) Functional and structural requirements for the internalization of distinct BCR-ligand complexes. *Eur. J. Immunol.* **36,** 3131–3145
28. Nashar, T. O., and Drake, J. R. (2005) The pathway of antigen uptake and processing dictates MHC class II-mediated B cell survival and activation. *J. Immunol.* **174,** 1306–1316
29. Gondré-Lewis, T. A., Moquin, A. E., and Drake, J. R. (2001) Prolonged antigen persistence within nonterminal late endocytic compartments of antigen-specific B lymphocytes. *J. Immunol.* **166,** 6657–6664
30. Boden, D., Pusch, O., Silbermann, R., Lee, F., Tucker, L., and Ramratnam, B. (2004) Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins. *Nucleic Acids Res.* **32,** 1154–1158
31. Geahlen, R. L., and Burg, D. L. (1994) The role of Syk in cell signaling. *Adv. Exp. Med. Biol.* **365,** 103–109
32. Winberg, G., Matskova, L., Chen, F., Plant, P., Rotin, D., Gish, G., Ingham, R., Ernberg, I., and Pawson, T. (2000) Latent membrane protein 2A of Epstein-Barr virus binds WW domain E3 protein-ubiquitin ligases that ubiquitinate B-cell tyrosine kinases. *Mol. Cell. Biol.* **20,** 8526–8535
33. Ikeda, A., Caldwell, R. G., Longnecker, R., and Ikeda, M. (2003) Itchy, a Nedd4 ubiquitin ligase, down-regulates latent membrane protein 2A activity in B-cell signaling. *J. Virol.* **77,** 5529–5534
34. Lupher, M. L., Jr., Rao, N., Lill, N. L., Andoniou, C. E., Miyake, S., Clark, E. A., Druker, B., and Band, H. (1998) Cbl-mediated negative regulation of the Syk tyrosine kinase. A critical role for Cbl phosphotyrosine-binding domain binding to Syk phosphotyrosine 323. *J. Biol. Chem.* **273,** 35273–35281
35. Rao, N., Ghosh, A. K., Ota, S., Zhou, P., Reddi, A. L., Hakezi, K., Druker, B. K., Wu, J., and Band, H. (2001) The non-receptor tyrosine kinase Syk is a target of Cbl-mediated ubiquitylation upon B-cell receptor stimulation. *EMBO J.* **20,** 7085–7095
36. Awasthi-Kalia, M., Schnetkamp, P. P., and Deans, J. P. (2001) Differential effects of filipin and methyl-β-cyclodextrin on B cell receptor signaling. *Biochem. Biophys. Res. Commun.* **287,** 77–82
37. Hou, P., Araujo, E., Zhao, T., Zhang, M., Massenburg, D., Veselits, M., Doyle, C., Dinner, A. R., and Clark, M. R. (2006) B cell antigen receptor signaling and internalization are mutually exclusive events. *PLoS Biol.* **4,** e200
38. Jacob, M., Todd, L., Sampson, M. F., and Puré, E. (2008) Dual role of Cbl links critical events in BCR endocytosis. *Int. Immunol.* **20,** 485–497
39. Azakir, B. A., and Angers, A. (2009) Reciprocal regulation of the ubiquitin ligase Itch and the epidermal growth factor receptor signaling. *Cell Signal* **21,** 1326–1336
40. Magnifico, A., Ettenberg, S., Yang, C., Mariano, J., Tiwari, S., Fang, S., Lipkowitz, S., and Weissman, A. M. (2003) WW domain HECT E3s target Cbl RING finger E3s for proteasomal degradation. *J. Biol. Chem.* **278,** 43169–43177