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CD2AP/SHIP1 Complex Positively Regulates Plasmacytoid Dendritic Cell Receptor Signaling by Inhibiting the E3 Ubiquitin Ligase Cbl

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Abstract

The human plasmacytoid dendritic cell (pDC) receptor BDCA2 forms a complex with the adaptor FcεR1γ to activate an ITAM-signaling cascade. BDCA2 receptor signaling negatively regulates the TLR7/9-mediated type 1 IFN responses in pDCs, which may play a key role in controlling self-DNA/RNA-induced autoimmunity. We report in this article that CD2-associated adaptor protein (CD2AP), which is highly expressed in human pDCs, positively regulates BDCA2/FcεR1γ receptor signaling. By immunoprecipitation and mass spectrometry analyses, we found that CD2AP bound to SHIP1. Knockdown of CD2AP or SHIP1 reduced the BDCA2/FcεR1γ-mediated ITAM signaling and blocked its inhibition of TLR9-mediated type 1 IFN production. Knockdown of CD2AP or SHIP1 also enhanced the ubiquitination and degradation of Syk and FcεR1γ that was mediated by the E3 ubiquitin ligase Cbl. This led us to discover that, upon BDCA2 cross-linking, the CD2AP/SHIP1 complex associated with Cbl and inhibited its E3 ubiquitin ligase activity. In human primary pDCs, cross-linking of the BDCA2/FcεR1γ complex induced the recruitment of the CD2AP/SHIP1/Cbl complex to the plasma membrane of pDCs, where it colocalized with the BDCA2/FcεR1γ complex. Therefore, CD2AP positively regulates BDCA2/FcεR1γ signaling by forming a complex with SHIP1 to inhibit the E3 ubiquitin ligase Cbl.

Plasmacytoid dendritic cells (pDCs), also known as type 1 IFN-producing cells, are specialized for rapidly producing massive amounts of type 1 IFN in response to nucleic acids derived from either virus or host cells (1, 2). pDCs selectively express TLR9 and TLR7, which recognize microbial DNA and RNA, respectively. Upon stimulation with ligands, TLR7/9 recruit MyD88 and activate a signaling cascade of IRAK4–IRAK1–TRAF6–IRF7, leading to the production of large amounts of type 1 IFN (3). Recognition of self-DNA/RNA by TLR7/9 in pDCs has been implicated in the development of autoimmune

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Disclosures

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diseases, such systemic lupus erythematosus (4–6). We and other investigators identified two pDC-specific receptor complexes: BDCA2/FcεR1γ and ILT7/FcεR1γ (7–9). Cross-linking of either BDCA2/FcεR1γ or ILT7/FcεR1γ receptor complex by Ab or natural ligand triggers an ITAM-mediated signaling cascade that turns off TLR7/9-mediated type 1 IFN responses in pDCs (10). These studies suggest that the pDC receptors negatively regulate TLR7/9 signaling, thereby limiting the magnitude and duration of type 1 IFN responses during a viral infection or type 1 IFN responses to self-DNA/RNA released by dead cells. Although the downstream signaling of BDCA2/FcεR1γ in pDCs has been studied extensively, the signaling components proximal to the membrane BDCA2/FcεR1γ receptor complex are unknown.

CD2-associated adaptor protein (CD2AP) belongs to the CIN85/CD2AP family that includes CIN85 and CD2AP; it contains three SH3 domains in the NH2 terminus, a proline-rich (P-rich) domain in the center region, and a coil-coiled domain in the COOH terminus. Members of the CIN85/CD2AP family regulate T cell activation, kidney glomeruli function, and apoptosis in neuronal cells (11). In particular, CD2AP and CIN85 can enhance the degradation of receptor tyrosine kinase (RTK) (11, 12). A recent study showed that CIN85 also enhances the ubiquitination and degradation of stimulated FcγRIIa mediated by Cbl (12). CIN85 and CD2AP associate with Cbl and enhance the degradation of RTK. The second SH3 domain of CD2AP binds specifically to Cbl upon stimulation of cell surface receptors (13). Upon ligand engagement, Cbl is recruited and mediates the ubiquitination of Syk and RTK that leads to their degradation, resulting in attenuation of receptor signals (14). Therefore, CIN85 and CD2AP appear to play important roles in the negative regulating of both RTK receptor signaling and FcγRIIa-mediated ITAM signaling by enhancing Cbl-mediated receptor ubiquitination and degradation. CD2AP was shown to be specifically expressed by human pDCs (15); however, its function has remained unknown.

In this study, we report that CD2AP binds SHIP1. Surprisingly, we found that the CD2AP/SHIP1 complex positively regulated BDCA2/FcεR1γ receptor signaling by inhibiting the E3 ubiquitin ligase Cbl.

Materials and Methods

Reagent and cells

CpG-A (2216) was purchased from Sigma-Genosys. Anti-p-Syk/Zap70, anti-SHIP1, and anti-Cbl Abs were purchased from Cell Signaling Technology; anti-p-VAV1 and anti-GFP Abs were purchased from Abcam; anti-β-actin and anti-hemagglutinin (HA) Abs and HA-agarose beads were purchased from Sigma-Aldrich; anti-Ub Ab, anti-Syk Ab, anti-CD2AP Ab and Alexa Fluor 647-conjugated anti-Cbl Ab were from Santa Cruz Biotechnology; and anti-phosphotyrosine (clone PY20) Ab was purchased from BD Biosciences. FcεR1γ Ab was from Upstate Biotechnology. The GFP-SHIP1 plasmid was kindly provided by Dr. Gerald Krystal (University of British Columbia, Vancouver, BC, Canada). The HA-Ub plasmid was provided by S.-C.S. ELISA kits for human IFN-α were from MabTech. Gen2.2 cells and HEK 293T cells were cultured, as described previously (9, 16). Human pDCs, myeloid dendritic cells, monocytes, T cells, B cells, and NK cells were isolated from the buffy coat from healthy donors, as described (9).

Reverse transcription and real-time PCR

Reverse transcription and real-time PCR was performed, as described previously (9). The primers for CD2AP were 5′-GGCATGGGAATGTAGCAAGT-3′ (forward) and 5′-GTGGATGTGGCTGAATTCCT-3′ (reverse). The primers for β-actin were 5′-

CTGGGACGACATGGAGAAAA-3' (forward) and 5'-
AAGGAAGGCTGGAAGAGTGC-3' (reverse).

Confocal microscopy

pDCs were isolated using the Plasmacytoid Dendritic Cell Isolation Kit (Miltenyi Biotec) and sorted as CD3⁻, CD14⁻, CD16⁻, CD56⁻, CD19⁻, CD20⁻, CD11c⁻. The purity of pDCs was checked by BDCA2 staining (purity > 95%). Fresh pDCs were placed on the cover slip, fixed, and permeabilized for staining. For the cross-linking assays, the purified pDCs were stained with BDCA2 Ab (mouse IgG1, produced in our laboratory) and cross-linked by adding the F(ab')₂ goat anti-mouse IgG secondary Ab for 2 or 20 min; cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin. Then, the cells were stained with Alexa Fluor-conjugated Abs or primary Abs, followed by Alexa Fluor-conjugated secondary Abs (Invitrogen). The stained cells were analyzed using a confocal microscope (Leica).

Immunoprecipitation analysis

HEK 293T cells were transfected with various plasmids, as indicated, with Lipofectamine (Invitrogen). Thirty-six hours after transfection, cells were lysed in 0.3% CHAPS, 10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM MgCl₂, and 1 mM EGTA with protease inhibitor. HA-tagged CD2AP or GFP-tagged SHIP1 was immunoprecipitated with HA-agarose or anti-GFP Ab, followed by Protein G beads. The beads were extensively washed with lysis buffer, and the proteins were separated by SDS-PAGE, followed by immunoblot analysis with appropriate Abs.

Knockdown experiments in Gen2.2 cells

The short hairpin RNA (shRNA) targeting CD2AP was constructed by cloning CCGGTAAGGACCTCAAAGAAACAGCTCGAGCTGTTTCT-TTGGAGTCTTATTTTTG into the pLKO.1 vector, as described (Addgene). The scrambled shRNA was from Addgene, and the shRNA targeting SHIP1 was from Open Biosystems. The lentiviral particles were produced according to the protocol described by Addgene. Gen2.2 cells were infected with shRNA lentiviral particles and selected in culture medium containing 1 mg/ml puromycin. For small interfering RNA (siRNA) knockdown experiments, the siRNA negative control (SIC001) and the siRNA targeting CD2AP (SASI_Hs02_00343664) and FcεR1γ (SASI_Hs01_00149039) were bought from Sigma-Aldrich. The siRNA targeting SHIP1 (GCCUCCAGAUCGGAAAUC) and Syk (ACCGUGGUG-UGAAAUAUCU) were synthesized from Sigma-Aldrich. The Gen2.2 cells were transfected with siRNA by Amaxa nucleofection; 48 h after siRNA transfection, Gen2.2 cells were collected and used for the experiments.

Calcium influx assay

The calcium influx assay was performed as described (7).

Ubiquitination assay

Gen2.2 cells with BDCA2 cross-linking or transfected HEK 293T cells were pretreated with 25 μM MG132 for 3 h and lysed in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% [v/v] Nonidet P-40, 0.5% [v/v] sodium deoxycholate, and 1 mM EDTA) supplemented with 4 mM Nethylmaleimide and protease inhibitor. Lysates were boiled for 5 min in the presence of 1% (v/v) SDS and diluted 10 times with RIPA buffer. Ubiquitinated Syk was analyzed by immunoprecipitation and detected by immunoblot analysis.

Statistical analysis

Data were analyzed statistically and are shown as mean \pm SD. The p values were calculated using an unpaired two-tailed Student *t* test; $p < 0.05$ was considered significant.

Results

CD2AP is highly expressed in pDCs, and knockdown of CD2AP reverses the BDCA2 cross-linking–induced inhibitory effect on type 1 IFN responses

During the past several years, our laboratory has identified several genes that are either specifically or highly expressed in human pDCs by analyzing a microarray database containing mRNA expression profiles of 12 different types of human leukocytes. We identified and confirmed that ILT7 and BDCA2 are pDC-specific receptors. We also found that CD2AP transcripts were highly expressed by pDCs (Fig. 1A). This result was confirmed by realtime PCR and immunoblotting analyses (Fig. 1B, 1C). These data confirm a previous report that CD2AP represents a maker for human pDCs (15). However, the function of CD2AP in pDC biology has been unknown.

CD2AP and its family member CIN85 share the same domain organization and were shown to enhance the degradation of RTK (11, 12). It was shown that CIN85 enhances the ubiquitination and degradation of FcγRIIa, which contains an ITAM motif (12). Previously, we showed that human pDCs selectively express BDCA2/FcεR1γ receptor, and BDCA2-mediated ITAM signaling inhibits TLR9-mediated type 1 IFN responses (9). Because both FcγRIIa and FcεR1γ receptors contain ITAM motifs, we investigated whether CD2AP plays a role in regulating BDCA2/FcεR1γ-mediated ITAM signaling.

We took advantage of Gen2.2 cells, a human pDC cell line that shares all of the key features of human primary pDCs (it is still not possible to manipulate gene expression in human primary pDCs) (16). Using siRNA, we knocked down CD2AP in Gen2.2 cells and stimulated the cells with CpG-A in the presence of plate-bound anti-BDCA2 Ab, which triggered ITAM signaling (9). The pDC cell line Gen2.2 produced high levels of IFN-α (6766 ± 447 pg/ml, $n = 3$) in response to CpG-A. BDCA2 cross-linking strongly inhibited IFN-α production (1852 ± 286 pg/ml, $n = 3$). Knockdown of CD2AP resulted in a 2-fold increase in IFN-α production in the presence of BDCA2 cross-linking (3639 ± 283 pg/ml $n = 3$). These data indicate that CD2AP plays a partial and positive role in BDCA2/FcεR1γ receptor signaling that negatively controls the TLR9-induced type 1 IFN responses in pDCs.

CD2AP binds to SHIP1, and CD2AP/SHIP1 complex is essential for BDCA2/FcεR1γ signaling in human pDCs

CD2AP has been implicated in the negative regulation of RTK signal transduction, and its family member CIN85 can negatively regulate ITAM-containing receptor FcγRIIa signaling by enhancing its degradation. Therefore, it is surprising that CD2AP plays a positive role in BDCA2/FcεR1γ receptor-mediated inhibition of type 1 IFN responses in pDCs. To investigate the molecular mechanism underlying the function of CD2AP in regulating pDC receptor signaling, we searched for CD2AP-binding proteins by immunoprecipitation with anti-CD2AP Ab in Gen2.2 cell lysate, followed by mass spectrometry analysis (Fig. 2A). Several previously described CD2AP-interacting proteins were identified, such as actin-capping protein, CIN85, and SH3BP1. In addition, we found novel binding partners, including SHIP1, vacuolar protein sorting-associated protein 33B, branched chain keto acid dehydrogenase E1, α polypeptide, and tripartite motif containing 21.

We focused on SHIP1 because of its relevance to receptor-mediated signaling: it is a hematopoietic phosphatase that hydrolyzes phosphatidylinositol-3,4,5-triphosphate to

phosphatidylinositol-3,4-bisphosphate, it binds to the phosphorylated FcεR1γ that contains the ITAM motif (17), and it binds to DAP12 and plays a negative role in ITAM signaling in preosteoclast cells (17, 18). First, we confirmed the binding of CD2AP and SHIP1 by coimmunoprecipitation in fresh isolated human pDCs (Fig. 2B). To investigate the function of CD2AP/SHIP1 in the regulation of BDCA2/FcεR1γ signaling, we knocked down CD2AP, SHIP1, as well as key molecules in ITAM signaling pathway—FcεR1γ and Syk, in Gen2.2 cells using siRNA. The knockdown cells were stimulated with CpG-A in the presence of plate-bound anti-BDCA2 Ab. We found that knockdown of CD2AP, SHIP1, FcεR1γ and Syk reversed the BDCA2-mediated inhibition of IFN-α production induced by CpG-A (Fig. 2C). These data indicate that CD2AP and SHIP1 are indispensable for the BDCA2 cross-linking-induced ITAM-signaling pathway. In addition, knockdown of CD2AP, SHIP1, FcεR1γ, and Syk marginally enhanced the CpG-A-induced type 1 IFN responses in pDCs, even in the absence of anti-BDCA2 Ab, suggesting that the BDCA2 receptor complexes or other receptors may constitutively activate ITAM signaling at a steady state (Supplemental Fig. 1). Previously, we showed that BDCA2 cross-linking activates the ITAM-signaling pathway in pDCs (9). We found that knockdown of CD2AP and SHIP1 inhibited total protein phosphorylation, as well as Syk kinase phosphorylation induced by BDCA2 cross-linking (Fig. 2D). The immunoblot results were quantified using ImageJ software (Supplemental Fig. 2A). Knockdown of CD2AP, SHIP1, FcεR1γ, and Syk in Gen2.2 cells did not fully recover the BDCA2 cross-linking-induced inhibition of type 1 IFN induction. This may be due to the incomplete knockdown in Gen2.2 cells. It is also possible that other molecules, such as CIN85, may play a redundant role. Altogether, these data suggest that CD2AP and SHIP1 play positive roles in BDCA2/FcεR1γ receptor complex-mediated ITAM signaling.

The first SH3 domain of CD2AP binds to the P-rich domain of SHIP1

CD2AP contains three SH3 domains, followed by one P-rich domain and one coil-coiled domain (Fig. 3A). SHIP1 contains a SH2 domain, a phosphatase domain, and one C-terminal P-rich domain (Fig. 3B). To map the domain interaction between CD2AP and SHIP1, we generated a panel of serial-deletion constructs of HA-CD2AP and GFP-SHIP1 and performed coimmunoprecipitation assays. We found that CD2AP bound to the P-rich domain of SHIP1 through its first SH3 domain (Fig. 3).

Knockdown of CD2AP or SHIP1 enhances the ubiquitination and degradation of FcεR1γ and Syk

To further confirm our results, we established CD2AP- and SHIP1-stable knockdown Gen2.2 cells by using shRNA-targeting lenti-viruses to analyze ITAM signaling triggered by BDCA2 cross-linking. As shown in Fig. 4A, knockdown of CD2AP or SHIP1 reduced the ITAM signaling triggered by BDCA2 cross-linking, which is characterized by a reduction in the activation of total protein phosphorylation, including the phosphorylation of Syk and Vav1. The immunoblot results were quantified using ImageJ software (Supplemental Fig. 2B). Calcium influx is another important cellular-activation event downstream of ITAM-mediated signaling. We found that knockdown of CD2AP or SHIP1 reduced the calcium influx triggered by cross-linking of the BDCA2/FcεR1γ complex (Fig. 4B). Consistent with the siRNA results, we found that knockdown of CD2AP or SHIP1 by shRNA increased the CpG-A-induced type 1 IFN responses by pDCs in the presence of BDCA2 cross-linking (Fig. 4C). All of these results suggest that knockdown of CD2AP or SHIP1 blocks BDCA2/FcεR1γ-mediated ITAM signaling that inhibits TLR9-mediated type 1 IFN responses.

We found reduced expression levels of Syk and FcεR1γ in the CD2AP- and SHIP1-stable knockdown Gen2.2 cells (Fig. 4D). Therefore, we checked the ubiquitination of Syk in the CD2AP and SHIP1 knockdown Gen2.2 cells. As shown in Fig. 4E, BDCA2 cross-linking

induced Syk ubiquitylation. However, knockdown of CD2AP or SHIP1 greatly enhanced total ubiquitination and K48-linked ubiquitination of Syk. These results suggest that the CD2AP/SHIP1 complex negatively regulates Syk ubiquitylation and degradation.

CD2AP/SHIP1 complex controls FcεR1γ and Syk degradation by inhibiting the E3 ubiquitin ligase activity of Cbl

Previous studies showed that Syk and FcεR1γ are targets of Cbl-mediated ubiquitylation (19, 20). Also, the second SH3 domain of CD2AP was found to bind specifically to Cbl (13). In CD2AP- and Cbl-transfected 293T cells, we found that CD2AP was associated with Cbl (Fig. 5A). Therefore, we examined whether CD2AP can bridge the association between SHIP1 and Cbl. As shown in Fig. 5B, overexpression of CD2AP enhanced the association of SHIP1 and Cbl. In addition, in Gen2.2 cells, BDCA2 cross-linking induced the association of the CD2AP/SHIP1 complex with Cbl (Supplemental Fig. 3). These results suggest that CD2AP mediates the association between SHIP1 and Cbl.

Because Syk ubiquitylation depends on Cbl, we tested whether CD2AP/SHIP1 complex regulates the E3 ubiquitin ligase activity of Cbl. In 293T cells, overexpression of Cbl enhanced Syk ubiquitylation, which was inhibited when CD2AP/SHIP1 was coexpressed in 293T cells (Fig. 5C). These results indicate that the CD2AP/SHIP1 complex functions as a negative regulator of Cbl E3 ubiquitin ligase.

CD2AP/SHIP1 complex and Cbl are recruited to the BDCA2/ FcεR1γ cross-linking–induced microcluster

Technically, it has been impossible to perform knockdown experiments in human primary pDCs. To establish evidence that CD2AP, SHIP1, and Cbl are related to the function of the BDCA2/ FcεR1γ receptor complex in human primary pDCs, we used confocal microscopy to examine the colocalization of CD2AP and SHIP1, as well as Cbl, in human primary pDCs before and after BDCA2 cross-linking. As shown in Fig. 6A, CD2AP was associated with SHIP1 at steady state, and CD2AP/SHIP1 microclusters were induced following BDCA2 cross-linking. In addition, we found that BDCA2 cross-linking induced the colocalization of CD2AP, Cbl, and FcεR1γ in the microclusters (Fig. 6B). These data suggest that CD2AP/SHIP1/Cbl represent the proximal signaling components and are recruited to the BDCA2/ FcεR1γ receptor complex after BDCA2 cross-linking.

Discussion

We and other investigators previously showed that BDCA2/FcεR1γ signaling triggers a BCR-like ITAM-mediated signaling cascade that negatively regulates the TLR7/9-mediated type 1 IFN responses in pDCs (9, 21). In this study, we showed that CD2AP, a signaling adaptor molecule, was highly expressed in human pDCs and formed a complex with SHIP1. The CD2AP/SHIP1 complex enhances BDCA2/FcεR1γ-induced ITAM signaling by inhibiting the E3 ubiquitin ligase activity of Cbl.

Although CIN85 and CD2AP were reported to play important roles in the negative regulation of both RTK receptor signaling and FcγRIIIa-mediated ITAM signaling by enhancing Cbl-mediated receptor ubiquitination and degradation (22–25), our study shows that the CD2AP/SHIP1 complex inhibits the Cbl-induced ubiquitination and degradation of FcεR1γ and Syk during BDCA2/FcεR1γ-induced ITAM signaling in pDCs. Our study suggests that CD2AP may play opposing roles, depending on the receptor types and cell types. In support of our hypothesis, a recent study showed that CD2AP deficiency leads to an early ubiquitination of nephrin and podocin after stimulation with fibroblast growth factor-4 in murine podocytes (26). It has been very well established that SHIP1 negatively

regulates ITAM signaling. However, it was also proposed that SHIP1 can play a positive role in immune receptor signaling (27). In murine platelets lacking SHIP1, glycoprotein VI-mediated dense granule secretion is potentiated, whereas protease-activated receptor-mediated dense granule secretion is inhibited (28). In this study, we provide more evidence that SHIP1 associates with CD2AP and plays a positive role in BDCA2/FcεR1γ-mediated ITAM signaling. Further, we demonstrated that the CD2AP/SHIP1 complex enhances BDCA2/FcεR1γ signaling by inhibiting the E3 ubiquitin ligase activity of Cbl to maintain the expression level of Syk and FcεR1γ.

In summary, our study demonstrates that the CD2AP/SHIP1 complex plays a very important role in maintaining the BDCA2 receptor-mediated ITAM signaling by negatively regulating Cbl-mediated ubiquitination and degradation of the activated Syk and FcεR1γ in pDCs. pDCs play a key role in antiviral immune responses by producing massive amounts of type 1 IFN upon viral infection. They have also been implicated in the development of autoimmune diseases, such as systemic lupus erythematosus, by sensing self-DNA/RNA (29). In addition, pDCs in the tumor microenvironment appear to have impaired ability to sense DNA/ RNA, possibly as the result of constitutive signaling of the pDC receptor ILT7/ FcεR1γ by its natural ligand BST2, which was shown to be expressed by tumor cells (10). Therefore, the identification of the CD2AP/SHIP1 complex as a key signaling component downstream of BDCA2/FcεR1γ provides a new therapeutic target for modulating the function of pDCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

CD2AP	CD2-associated adaptor protein
HA	hemagglutinin
pDC	plasmacytoid dendritic cell
P-rich	proline-rich
RTK	receptor tyrosine kinase
shRNA	short hairpin RNA

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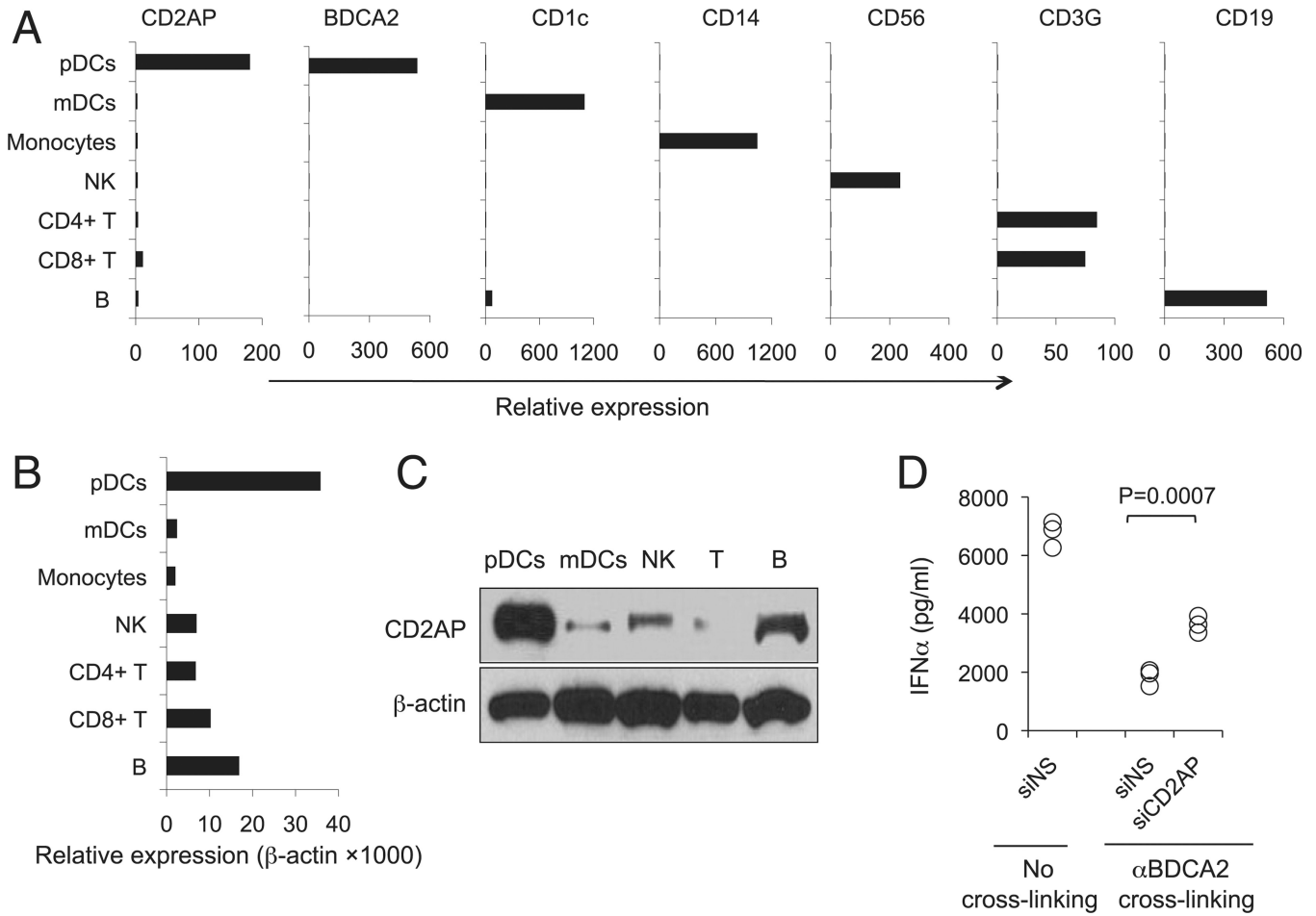


FIGURE 1. CD2AP is highly expressed in pDCs and is indispensable for BDCA2 signaling. **(A)** The relative expression level of CD2AP from the human expression microarray database. The relative expression of each gene was compared by plotting the values extracted from the gene expression database. A value < 1 indicated the absence of gene expression. **(B)** Human pDCs, myeloid dendritic cells (mDCs), monocytes, NK cells, T cells, and B cells were isolated from PBMCs, and total RNA was purified and reverse transcribed. The cDNAs were subjected to real-time PCR analysis, and the expression of CD2AP was normalized to the level of β-actin, whose value was multiplied by 1000. **(C)** Human pDCs, as well as other major immune cell types in human PBMCs, were isolated, and protein extracts of 1×10^5 cells/lane were separated by SDS-PAGE and subjected to immunoblot analysis using anti-CD2AP Ab. β-actin was used as a loading control. **(D)** Using siRNA, CD2AP was knocked down in Gen2.2 cells, and the cells were stimulated with $1 \mu\text{M}$ CpG-A in the presence of plate-bound anti-BDCA2 Ab for 24 h. IFN-α in the supernatants was determined by ELISA. Each circle indicates an independent experiment.

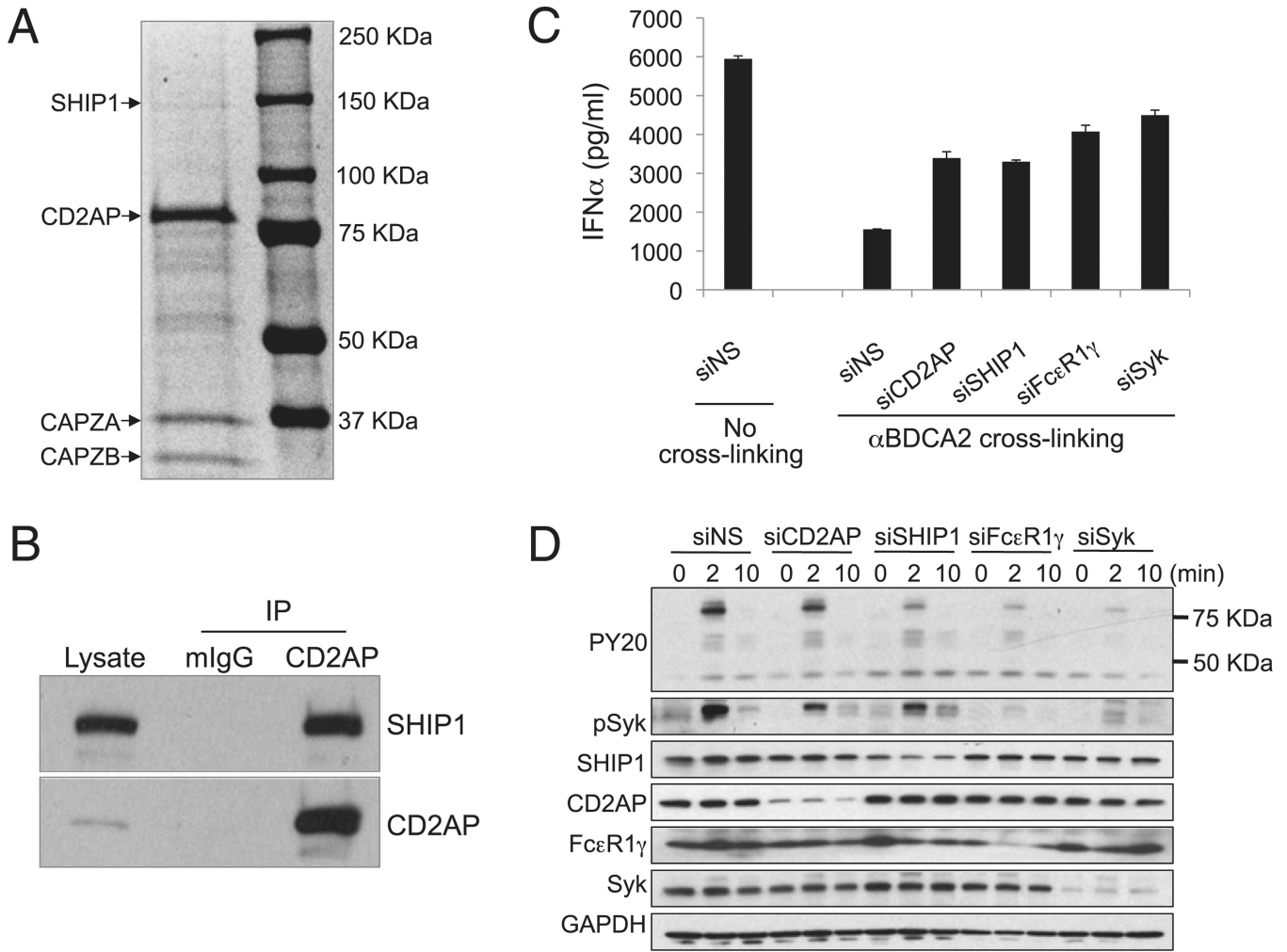


FIGURE 2. CD2AP binds to SHIP1 and the CD2AP/SHIP1 complex is needed for BDCA2 signaling. **(A)** Coomassie blue staining of CD2AP-binding proteins purified by immunoprecipitation of CD2AP from Gen2.2 cell lysate. Proteins identified by mass spectrometry are as indicated. **(B)** CD2AP was immunoprecipitated from human primary pDC lysate, and CD2AP-binding proteins were subjected to SDS-PAGE and subsequent immunoblot analysis by anti-SHIP1 Ab. **(C)** CD2AP, SHIP1, Fc ϵ R1 γ , and Syk were knocked down using siRNA in Gen2.2 cells. The nontargeting siRNA (siNS) was used as a control. The siRNA knockdown Gen2.2 cells were stimulated with 1 μ M CpG-A in the presence of plate-bound BDCA2. The IFN- α level in the supernatant was measured by ELISA. **(D)** The siRNA knockdown Gen2.2 cells were cross-linked using anti-BDCA2 Ab for 2 or 10 min. The total protein phosphorylation and phosphorylation of Syk, as well as total protein levels of CD2AP, SHIP1, Fc ϵ R1 γ , and Syk, were analyzed by immunoblotting. β -actin was used as a loading control. Data are representative of three independent experiments.

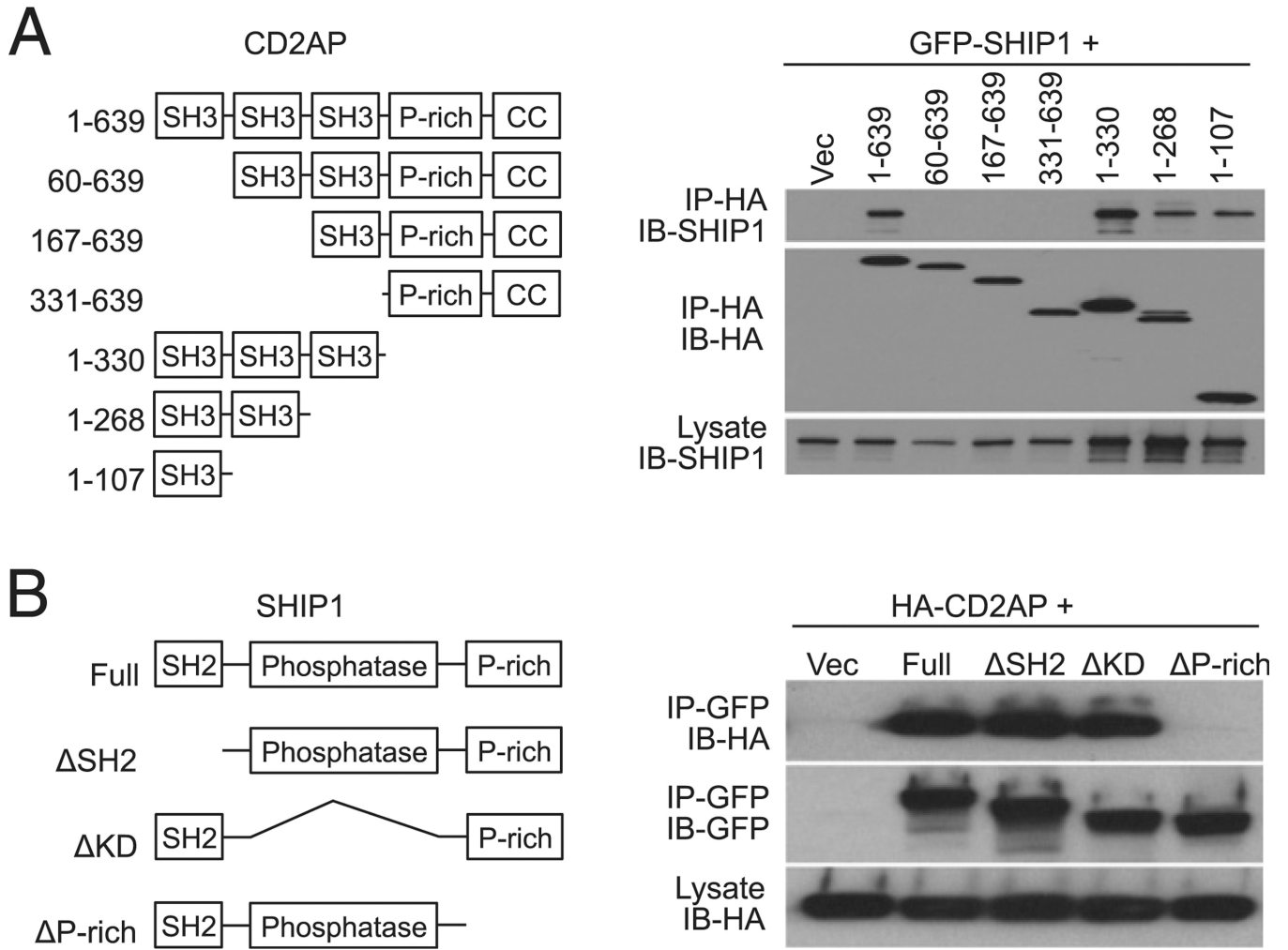


FIGURE 3.

The first SH3 domain of CD2AP binds to the P-rich domain of SHIP1. **(A)** Lysates from 293T cells transfected with full-length GFP-SHIP1 and HA-tagged full-length CD2AP or deletion mutants of CD2AP were immunoprecipitated with HA-agarose beads, followed by immuno-blotting with anti-SHIP1 Ab or with anti-HA Ab. **(B)** Lysates from 293T cells transfected with HA-tagged full-length CD2AP and full-length or deletion mutants of GFP-SHIP1 were immunoprecipitated with anti-GFP Ab, followed by immunoblotting with anti-HA tag Ab or anti-GFP Ab.

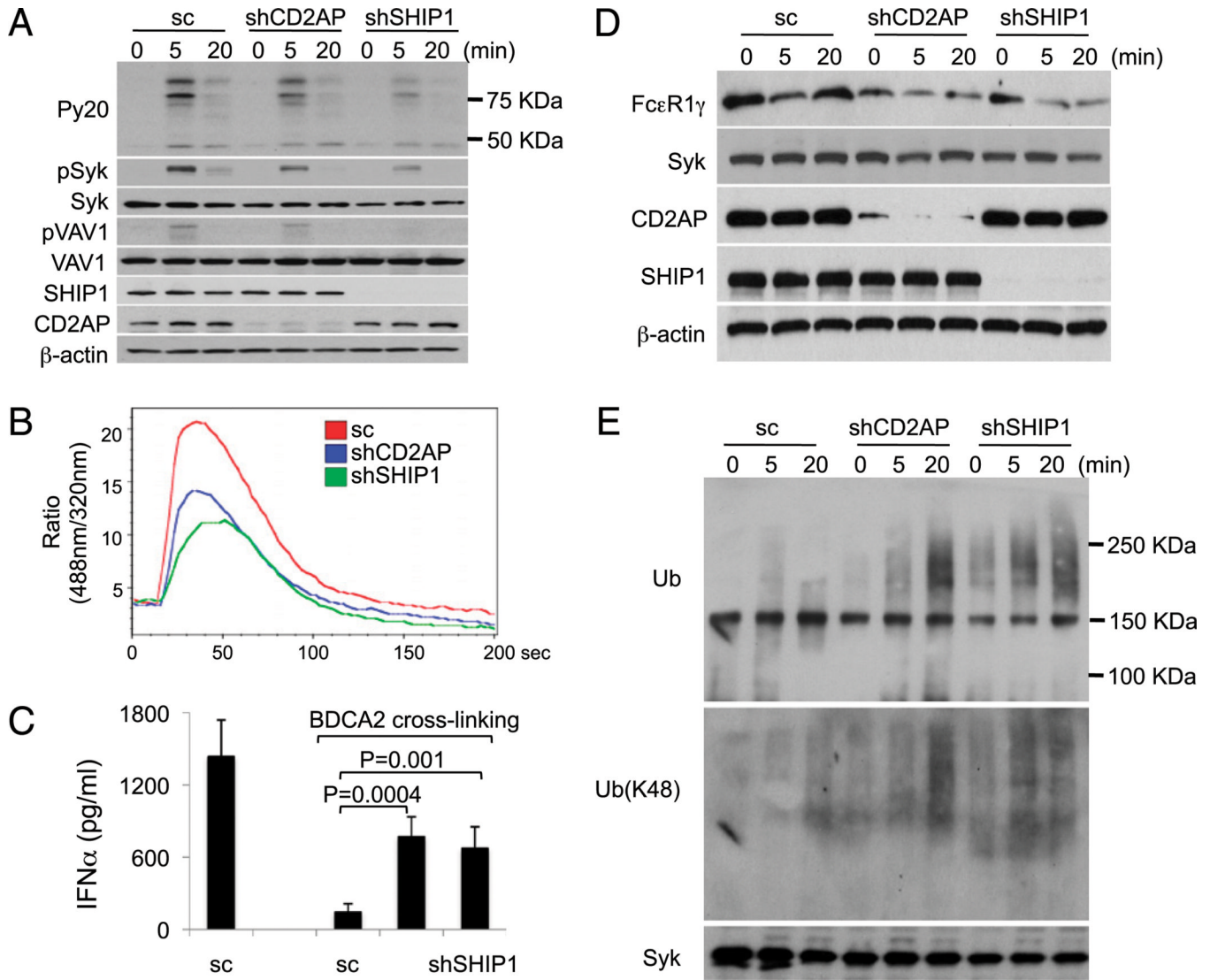


FIGURE 4. Knockdown of CD2AP or SHIP1 using shRNA reduces ITAM signaling and enhances the degradation and ubiquitination of Syk. **(A)** CD2AP knockdown Gen2.2 cells (shCD2AP), SHIP1 knockdown Gen2.2 cells (shSHIP1), or scramble shRNA knockdown Gen2.2 cells (sc) were cross-linked using anti-BDCA2 Ab for 5 or 20 min. The total protein phosphorylation and phosphorylation of Syk, Vav1, and SHIP1, as well as total protein levels of CD2AP and SHIP1, were analyzed by immunoblotting. β -actin was used as a loading control. **(B)** shRNA knockdown Gen2.2 cells were cross-linked by BDCA2, and the calcium influx was analyzed by FACS. **(C)** shRNA knockdown Gen2.2 cells were stimulated with 1 mM CpG-A in the presence of plate-bound anti-BDCA2 Ab. The IFN- α level in the supernatant was measured by ELISA. Data are representative of six independent experiments. **(D)** shRNA knockdown Gen2.2 cells were cross-linked by BDCA2 for 5 or 20 min. Protein levels of Fc ϵ R1 γ , Syk, CD2AP, and SHIP1 were analyzed by immunoblotting. β -actin was used as a loading control. **(E)** shRNA knockdown Gen2.2 cells were cross-linked using anti-BDCA2 Ab for 5 or 20 min. Syk was immunoprecipitated, resolved by SDS-PAGE, and immunoblotted with anti-Ub Ab and anti-Ub (K48-specific) Ab.

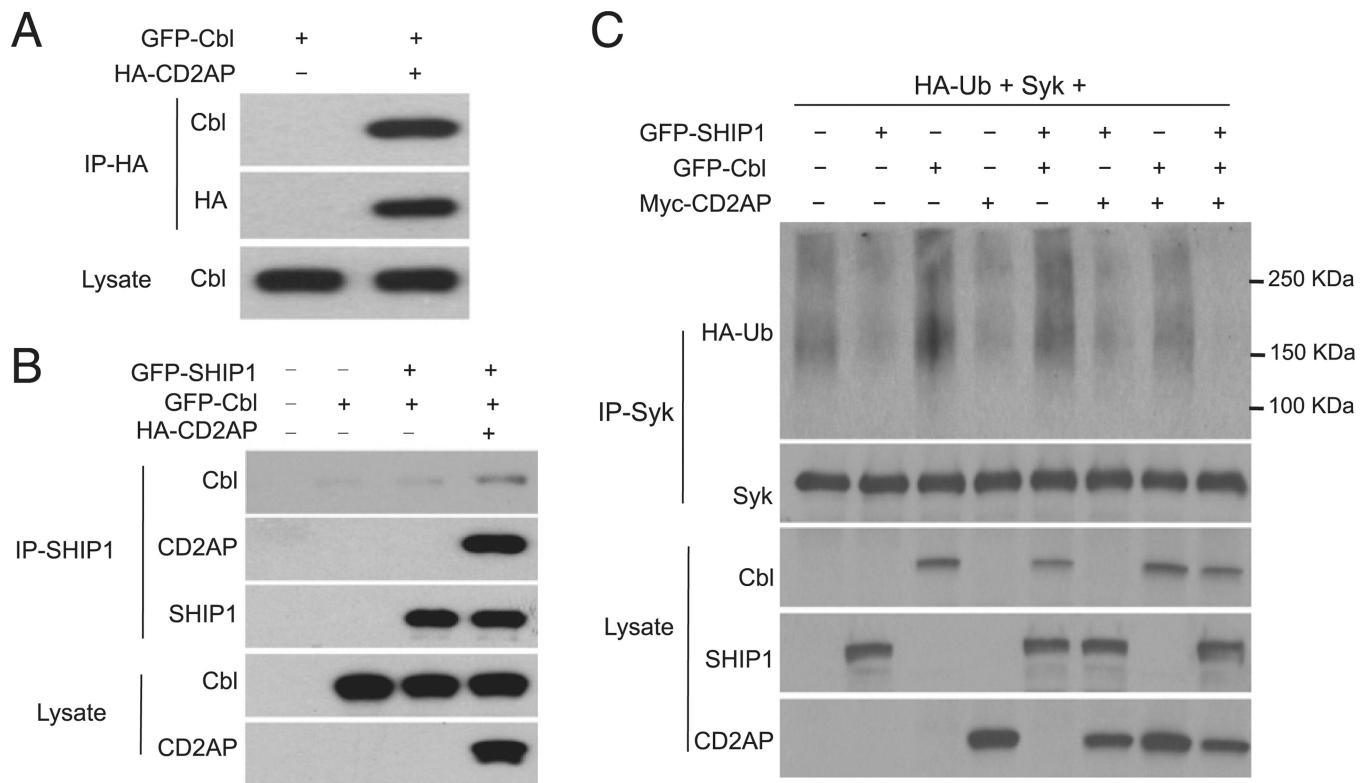


FIGURE 5. CD2AP mediates the association of SHIP1 and Cbl, and the CD2AP/SHIP1 complex inhibits the E3 ubiquitin ligase activity of Cbl. **(A)** 293T cells were transfected with GFP-Cbl together with HA-CD2AP, 36 h after transfection, anti-HA-CD2AP immunoprecipitates from the lysate protein were immunoblotted with anti-Cbl. Aliquots of cell lysates were immunoblotted directly with an anti-Cbl Ab to visualize GFP-Cbl. **(B)** 293T cells were transfected with GFP-Cbl, together with HA-CD2AP and GFP-SHIP1; 36 h after transfection, SHIP1 was immunoprecipitated and immunoblotted with anti-Cbl and anti-HA Abs. Aliquots of cell lysates were immunoblotted directly with anti-Cbl and anti-HA Abs to visualize GFP-Cbl and HA-CD2AP. **(C)** 293T cells were transfected with the indicated expression plasmids together with HA-ubiquitin and Syk plasmids; 24 h after transfection, cells were incubated with 25 mM MG132 for 3 h. Syk was immunoprecipitated from the cell lysates and immunoblotted with anti-HA, anti-Syk, anti-SHIP1, and anti-Myc Abs. Aliquots of cell lysates were immunoblotted directly with indicated Abs. Data are representative of three independent experiments.

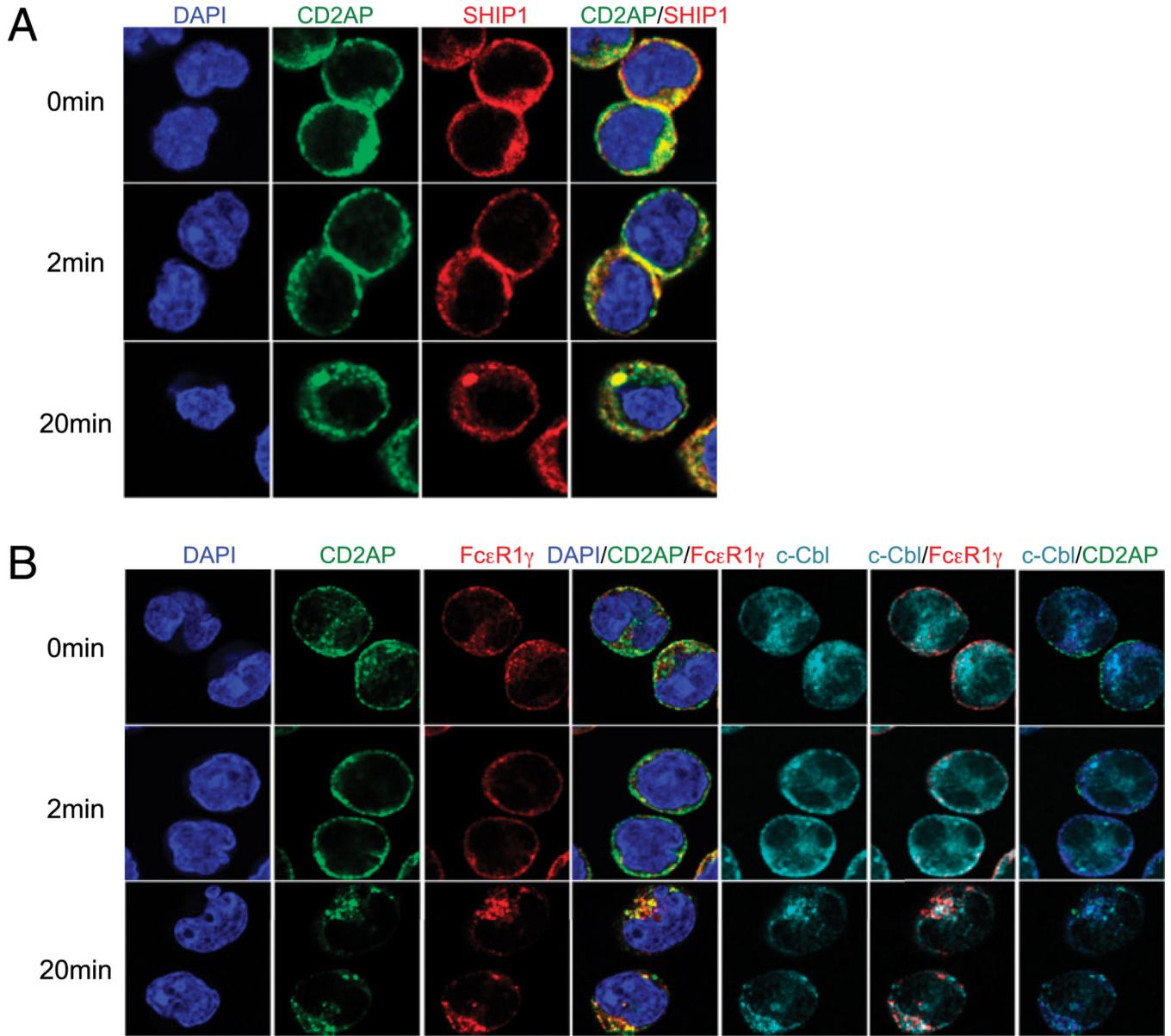


FIGURE 6. The CD2AP/SHIP1 complex and Cbl are recruited to BDCA2 and FcεR1γ complex after BDCA2 cross-linking in human primary pDCs. **(A)** pDCs were cross-linked using BDCA2 mAb for 0, 2, or 20 min. The pDCs were stained with anti-CD2AP and anti-SHIP1 Abs, as described in Materials and Methods. **(B)** pDCs were cross-linked using BDCA2 mAb for 0, 2, or 20 min. The pDCs were stained with anti-CD2AP, anti-FcεR1γ, and Alexa Fluor 647-conjugated anti-Cbl Abs. Images were taken using a Leica confocal microscope. Original magnification $\times 1260$.