Brief Definitive Report

Physical and Functional Association of the *cbl* Protooncogene Product with an Src-Family Protein Tyrosine Kinase, p53/56^{lyn}, in the B Cell Antigen Receptor–mediated Signaling

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Summary

To identify novel signal transducers involved in signaling mediated by the Src-family protein tyrosine kinases (PTKs), we used a yeast two-hybrid system with a probe corresponding to the regulatory region of p56^{lyn}, a member of Src-family PTKs. One of the isolated clones contained the COOH-terminal 470 amino acid residues of p120$^{c\text{-}cbl}$, the product of the cellular homologue of the *v-cbl* retroviral oncogene. p120$^{c\text{-}cbl}$ is a cytoplasmic protein with nuclear protein–like motifs. Here we show in vivo association of p120$^{c\text{-}cbl}$ with p53/56^{lyn}. After stimulation of the B cell antigen receptor (BCR), p120$^{c\text{-}cbl}$ was rapidly tyrosine phosphorylated. Studies with *lyn*- or *syk*-negative chicken B cells demonstrated that p53/56^{lyn}, but not p72^{syk}, was crucial for tyrosine phosphorylation of p120$^{c\text{-}cbl}$ upon stimulation of the BCR. We also show the importance of p59^{fyn} in tyrosine phosphorylation of p120$^{c\text{-}cbl}$ in the T-cell receptor–mediated signaling, using *fyn*-overexpressing T cell hybridomas and splenic T cells from *fyn*-deficient mice. These results suggest that p120$^{c\text{-}cbl}$ is an important substrate of Src-family PTKs in the intracellular signaling mediated by the antigen receptors.

The Src-family protein tyrosine kinases (PTKs) are intracytoplasmic PTKs that are generally localized on the inner part of the plasma membrane via myristylation. Accumulating evidence shows that these PTKs transduce signals from specific cell surface receptors, including the B cell antigen receptor (BCR) (1–5). Stimulation of the BCR initiates a series of intracellular signaling events, such as protein tyrosine phosphorylation, phosphatidylinositol turnover, Ras activation, and Ca^{2+} mobilization (5). Since Src-family PTKs are associated both physically and functionally with the BCR, they have been thought to be critically important in triggering the BCR-mediated signaling. In B cell activation, several significant PTK substrates have been identified, including components of the BCR complex, phospholipase C-γ (PLC-γ), phosphatidyl inositol-3 (PI-3) kinase, Ras GTPase-activating protein (GAP), GAP-associated protein p62, Vav, HS1, and Shc (2, 5–7). Many of these PTK substrates have been reported to be physically associated with Src-family PTKs in various cell types (2, 6, 8). Thus, it is proper to search for Src-family PTK-interacting proteins to identify and discern the molecules that are involved in B cell activation.

The viral oncogene *v-cbl* was originally discovered as the transforming gene of the murine Cas NS-1 retrovirus, which induces pre-B lymphomas and myelogenous leukemias (9). The transforming gene product p100$^{gag\text{-}v\text{-}cbl}$ is a Gag-fused protein containing the NH_2-terminal 355–amino acid sequence of the *c-cbl* gene product (9, 10). Although the function of p120$^{c\text{-}cbl}$ has not yet been uncovered, it possesses the following protein motifs (10): (*a*) a YY-1 transcription factor–like basic region; (*b*) a nuclear localization signal; (*c*) a RING finger; (*d*) two regions rich in acidic amino acids; (*e*) a stretch of 208 amino acids rich in Pro, Ser, and Thr; and (*f*) a putative leucine zipper. Expression of *c-cbl* is high in thymus and testis (11). It is also expressed in spleen at the lower level. p120$^{c\text{-}cbl}$ is a cytoplasmic protein, whereas p100$^{gag\text{-}v\text{-}cbl}$ localizes both in cytoplasm and nucleus (12). Recent evidence shows that p120$^{c\text{-}cbl}$ becomes tyrosine phosphorylated upon TCR stimulation (13). In addition, the SH3 domain of various signaling molecules is shown to bind to p120$^{c\text{-}cbl}$ in vitro (14).

One of the Src-family PTKs, p53/56^{lyn}, participates in the intracellular signaling mediated by the BCR as well as the FcεRI and FcγRI (1–5). In this study, we searched for novel p56^{lyn}-associated molecules to characterize further the signaling pathway of p53/56^{lyn}. Using a yeast two-

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hybrid system with the regulatory region of $p56^{lyn}$ as a probe, we identified the *cbl* protooncogene product as a $p53/56^{lyn}$–interacting molecule. Here we report that the Src-family PTKs $p53/56^{lyn}$ and $p59^{fyn}$ are crucially important in tyrosine phosphorylation of $p120^{c\text{-}cbl}$ upon stimulation of the lymphocyte antigen receptors.

Materials and Methods

Yeast Two-Hybrid Screening. The sequence encoding the NH_2-terminal 235 amino acids of $p56^{lyn}$ (15) was cloned in-frame into pAS2. The resultant plasmid, designated as pAS2-Lyn(1-235), expresses the regulatory region of $p56^{lyn}$ fused to the GAL4 DNA-binding domain, as was confirmed by Western blotting. A random-primed adult rat cDNA library was synthesized using the Time Saver cDNA Synthesis Kit (Pharmacia, Uppsala, Sweden) and cloned into pGAD424 (Clontech Laboratories Inc., Palo Alto, CA). For yeast two-hybrid screening, the bait plasmid pAS2-Lyn(1-235) was cointroduced with the library plasmids into the yeast strain Y190 by a lithium acetate method. Transformants (2×10^6 cells) were plated on dropout media lacking Trp, Leu, and His and containing 40 mM of 3-amino-1,2,4-triazole and analyzed as described previously (16). DNA sequences were determined using the *Bca*Best Sequencing Kit (Takara Shuzo Corp., Shiga, Japan).

Cell Culture. WEHI-231, DT40, and HBC21.7.31 (HBC) cells were maintained as described previously (2, 17, 18). *lyn*-negative and *syk*-negative DT40 cells were established by gene targeting (17). HBC21.7.31 is a class II–restricted murine helper T cell hybridoma. Establishment of *fyn*-overexpressing HBC21.7.31 cells, named N17 cells, was reported previously (18). Splenic T cells were prepared from wild-type and *fyn*-deficient mice (19) by using nylon wool columns (20).

Antibodies. Polyclonal rabbit Abs against $p120^{c\text{-}cbl}$ (C-15), $p55^{blk}$ (K-23), and $p56/59^{hck}$ (N-30) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse mAbs against $p59^{fyn}$ (301), $p53/56^{lyn}$ (Lyn-8), and $p72^{syk}$ (101) were from Wako Pure Chemical Industries (Osaka, Japan). Mouse anti-$p56^{lck}$ mAb was from Y. Koga (Tokai University, Kanagawa, Japan) (21). PY20, an antiphosphotyrosine mAb, was from ICN (Irvine, CA). For cross-linking of the lymphocyte antigen receptors, the following Abs were used: hamster anti–mouse CD3ε, 145-2C11 (gift of T. Katagiri, Kitasato University, Tokyo, Japan); rabbit anti–hamster IgG (Cappel Laboratories, West Chester, PA); goat anti–mouse IgM (Sigma Chemical Co., St. Louis, MO); and goat anti–chicken IgM (Bethyl Laboratories Inc., Montgomery, TX).

Coimmunoprecipitation and Kinase Assay. Rat spleens were homogenized in 1% digitonin buffer (1). The lysates were incubated with anti-$p120^{c\text{-}cbl}$ at 4°C for 1 h, and the immune complexes were precipitated with protein A–Sepharose (Pharmacia). The immunoprecipitates were washed five times with 1% digitonin buffer, three times with kinase buffer (20 mM Hepes–NaOH, 10 mM $MgCl_2$, 10 mM $MnCl_2$, pH 7.4) and then subjected to the kinase reaction in the presence of [γ-^{32}P]ATP (Amersham Corp., Arlington Heights, IL) at 25°C for 30 min. The reaction complexes were dissociated with solubilizing buffer (1). The supernatants were reimmunoprecipitated with the second Abs as shown in Fig. 1. The immunoprecipitates were separated by 8.5% SDS-PAGE, and phosphorylated proteins were visualized by autoradiography.

Cell Stimulation. Stimulation of B cells with anti-IgM was performed basically as described (2). In the case of HBC cells and mouse splenic T cells, the cells were incubated with the hamster anti–mouse CD3ε. After being washed twice, they were stimulated by rabbit anti–hamster IgG (18).

Immunoprecipitation and Western Blotting. The cells were lysed with 1 ml of ice-cold TNE buffer and then subjected to immunoprecipitation as described (2). The immunoprecipitates or the whole-cell lysates were separated by 7.5% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories, Richmond, CA), and then blocked as described (6). The filter was probed with an appropriate first Ab, followed by either horseradish peroxidase–conjugated Ab (Amersham Corp.) or alkaline phosphatase–coupled Ab (Promega Corp., Madison, WI) to rabbit IgG or to mouse IgG. The signals were detected either with Renaissance (DuPont, Boston, MA) or with the Protoblot NBT and BCIP color development system (Promega Corp.). For reprobing, the filters were stripped and then reblocked according to the manufacturer's protocol (Amersham Corp.).

Results and Discussion

Identification of $p120^{c\text{-}cbl}$ as a Novel $p53/56^{lyn}$-associated Protein. For identification of proteins that associate with $p53/56^{lyn}$, we used a yeast two-hybrid system. A probe corresponding to the unique, SH3, and SH2 regions of $p56^{lyn}$ was fused to the GAL4 DNA binding domain. As a prey, a random-primed adult rat cDNA library was generated using pGAD424, a vector that produces cDNA-encoded proteins fused to the GAL4 transcriptional activation domain. By screening 2×10^6 yeast transformants, we isolated five

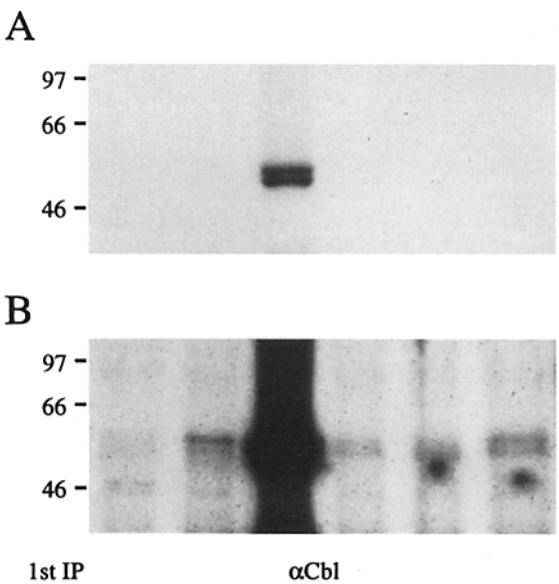

Figure 1. Physical association of $p120^{c\text{-}cbl}$ with $p53/56^{lyn}$. The 1% digitonin lysates of rat spleens were immunoprecipitated with anti-$p120^{c\text{-}cbl}$ Ab, and the immunoprecipitates were subjected to kinase reaction in the presence of [γ-^{32}P]ATP. The reaction products were dissociated with solubilizing buffer, and then the extracts were reimmunoprecipitated with the second Abs (*ReIP*). The reimmunoprecipitates were separated by 8.5% SDS-PAGE. *B* shows a longer exposure of *A*. Positions and sizes (kD) of standard protein markers are indicated.

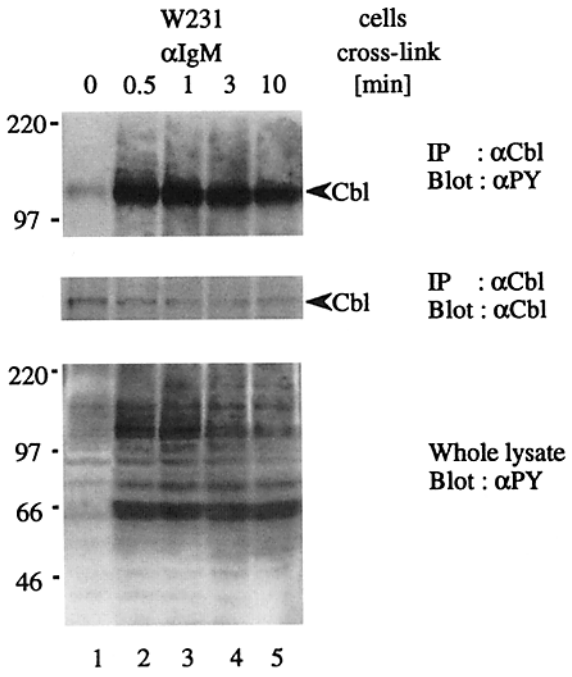

Figure 2. Rapid tyrosne phosphorylation of p120$^{c\text{-}cbl}$ upon BCR stimulation. 10^7 WEHI-231 cells were stimulated with 10 μg of anti–mouse IgM for the indicated periods and lysed with TNE buffer. (*Top*) Anti-p120$^{c\text{-}cbl}$ immunoprecipitates from 10^7 cells were tested for phosphotyrosine by Western blotting with PY20. (*Middle*) The same filter used in the top panel was reprobed with anti-p120$^{c\text{-}cbl}$. (*Bottom*) Whole-cell lysates from 2.5×10^5 cells were tested for phosphotyrosine. Positions and sizes (kD) of standard protein markers are indicated. W231 indicates WEHI-231.

positive clones. Nucleotide sequencing revealed that the cDNA insert of one of the clones coded for the COOH-terminal 470 amino acids of p120$^{c\text{-}cbl}$. Since *c-cbl* and *lyn* are both expressed in the spleen, we examined whether p53/56^{lyn} was physically associated with p120$^{c\text{-}cbl}$ in the cells of the spleen. Coimmunoprecipitation experiments showed that p53/56^{lyn} was predominantly present in the anti-p120$^{c\text{-}cbl}$ immunoprecipitates from 1% digitonin lysates of the rat spleen (Fig. 1 *A*). A low level of p59^{fyn} was also detected in the immunoprecipitates. The level of p55^{blk}, p56^{lck}, p56/p59^{hck}, or p72^{syk} was much lower in the anti-p120$^{c\text{-}cbl}$ immunoprecipitates (Fig. 1 *B*). However, the level of expression of *lyn* is predominant in the spleen as compared with the other *src*-family members (data not shown). Conversely, p120$^{c\text{-}cbl}$ was present in the anti-p53/56^{lyn} immunoprecipitates from WEHI-231 cells, which confirmed physical association between p120$^{c\text{-}cbl}$ and p53/56^{lyn} (see Fig. 3). The isolated cDNA clone included the sequence coding for a stretch of Pro/Ser/Thr–rich 208 amino acids in p120$^{c\text{-}cbl}$. Consistent with the finding that proline-rich sequences physically interact with the SH3 domain (22), p120$^{c\text{-}cbl}$ binds to the SH3 domain of various proteins in vitro (13, 14). In addition, p120$^{c\text{-}cbl}$ associates in vivo with p47^{nck} (14) and p150$^{c\text{-}abl}$ (12), both of which contain the SH3 domain. The physical association between p120$^{c\text{-}cbl}$ and p53/56^{lyn} would be at least partly due to the interaction of the proline-rich domain in p120$^{c\text{-}cbl}$ with the SH3 domain in p53/56^{lyn}, but further dissection of domain(s) involved in the in vivo association would be required.

Critical Role of p53/56^{lyn} in Rapid Tyrosine Phosphorylation of p120$^{c\text{-}cbl}$ upon BCR Stimulation. Previous studies have shown that p53/56^{lyn} is activated by stimulation of the BCR (2). To examine the possibility that p120$^{c\text{-}cbl}$ was a substrate of p53/56^{lyn} in the BCR-mediated signaling, we evaluated tyrosine phosphorylation of p120$^{c\text{-}cbl}$ after activation through the BCR. p120$^{c\text{-}cbl}$ was rapidly tyrosine phosphorylated, being maximally phosphorylated within 30 s upon BCR stimulation on WEHI-231 cells (Fig. 2, *top*). The amount of p120$^{c\text{-}cbl}$ did not change over the assay period (Fig. 2, *middle*). Since p120$^{c\text{-}cbl}$ was tyrosine phosphorylated upon TCR stimulation (13), p120$^{c\text{-}cbl}$ was suggested to be a common target of PTKs participating in the antigen receptor–mediated signaling in both B cells and T cells.

Tyrosine phosphorylation of signaling molecules can promote their association with Src-family PTKs, which is at least partially mediated by the SH2 domain of Src-family PTKs (6, 8). Since tyrosine-phosphorylated p120$^{c\text{-}cbl}$ binds to a variety of SH2 domains in vitro (13), the association between p120$^{c\text{-}cbl}$ and Src-family PTKs could increase upon BCR stimulation. The amount of p120$^{c\text{-}cbl}$ in p53/56^{lyn} immunoprecipitates, however, did not change upon stimulation of the BCR on WEHI-231 cells (Fig. 3). Thus, tyrosine phosphorylation of p120$^{c\text{-}cbl}$ might recruit other SH2-containing proteins to p120$^{c\text{-}cbl}$. It is also possible that tyrosine-phosphorylated p120$^{c\text{-}cbl}$ may signal in an SH2-independent manner.

Stimulation of the BCR activates three classes of nonreceptor PTKs, namely, PTKs of the Src-family (p53/56^{lyn} and

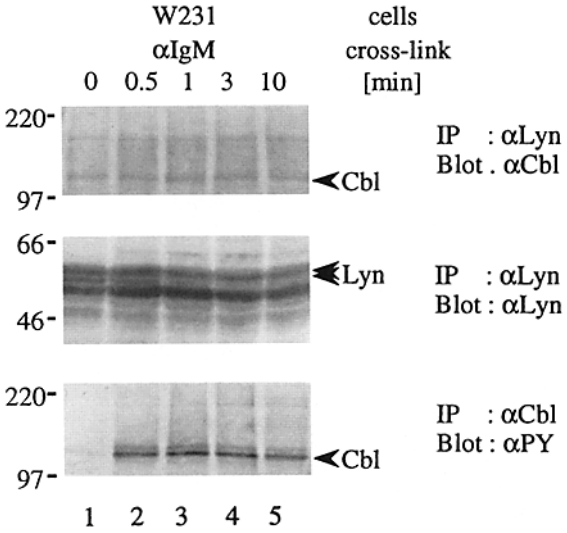

Figure 3. Constitutive association between p120$^{c\text{-}cbl}$ and p53/56^{lyn} upon stimulation of the BCR on WEHI-231 cells. 10^7 WEHI-231 cells were stimulated with 10 μg of anti–mouse IgM for the indicated periods. Anti-p53/56^{lyn} immunoprecipitates from 5×10^6 cells were tested for the amount of p120$^{c\text{-}cbl}$ (*top*) and for the amount of p53/56^{lyn} (*middle*). (*Bottom*) Anti-p120$^{c\text{-}cbl}$ immunoprecipitates from 5×10^6 cells were tested for phosphotyrosine. Positions and sizes (kD) of standard protein markers are indicated. W231 indicates WEHI-231.

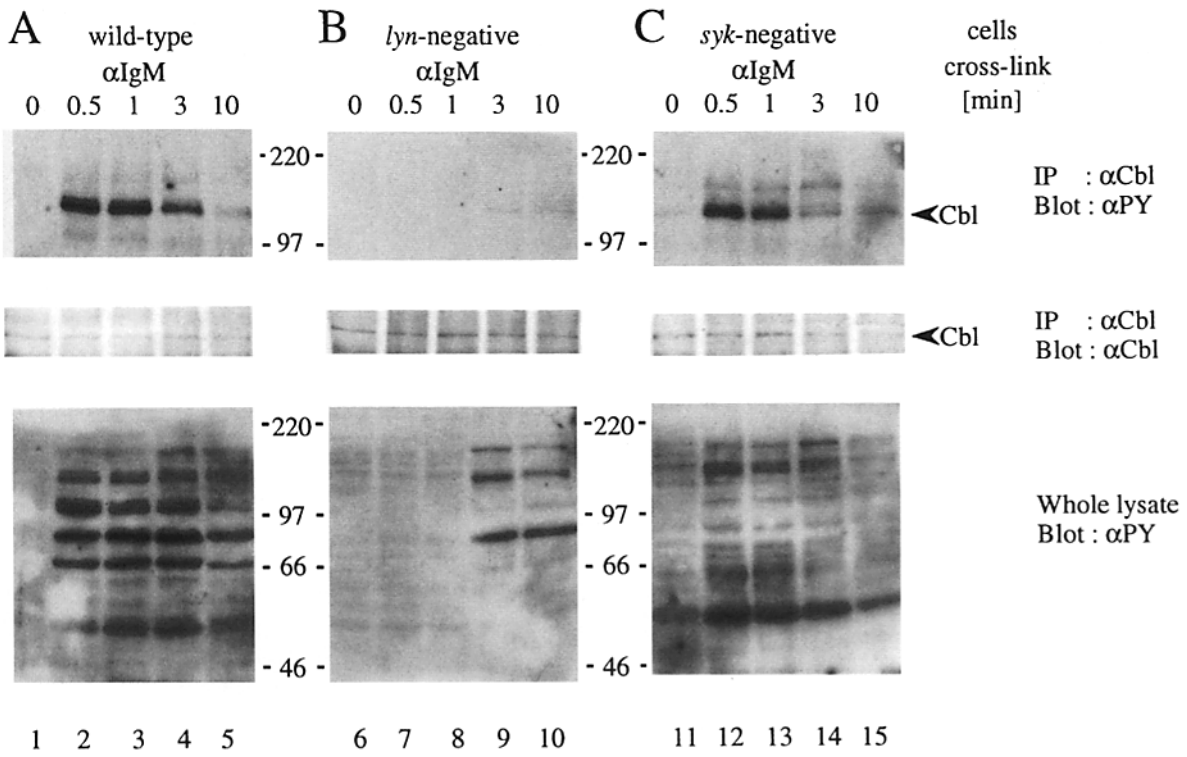

Figure 4. Tyrosine phosphorylation of p120$^{c\text{-}cbl}$ in wild-type, *lyn*-negative, and *syk*-negative DT40 cells upon stimulation of the BCR. Wild-type (*A*), *lyn*-negative (*B*), and *syk*-negative (*C*) DT40 cells (8×10^6) were stimulated with 10 μg of anti-chicken IgM for the indicated time. (*Top*) Anti-p120$^{c\text{-}cbl}$ immunoprecipitates from 8×10^6 cells were tested for phosphotyrosine. (*Middle*) The same filters used in the top panel were reprobed with anti-p120$^{c\text{-}cbl}$. (*Bottom*) Whole-cell lysates from 2×10^5 cells were tested for phosphotyrosine. Positions and sizes (kD) of standard protein markers are indicated.

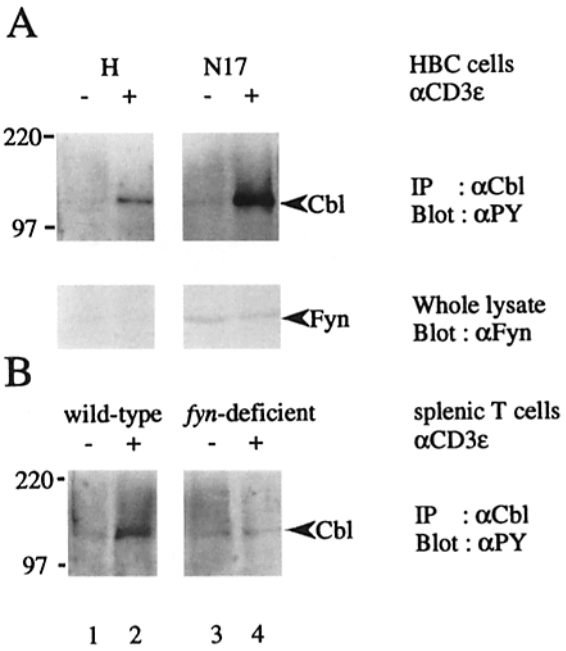

Figure 5. Importance of p59^{fyn} in tyrosine phosphorylation of p120$^{c\text{-}cbl}$ in the TCR-mediated signaling. (*A*) Parental and *fyn*-overexpressing HBC cells (10^7) were incubated with (+) or without (−) hamster anti-mouse CD3ε (145-2C11; supernatant of hybridoma, diluted 1:2) on ice for 1 h. After washing, the cells were then stimulated with 1 μg of anti-hamster IgG at 37°C for 1 min. The parental cells are designated as H cells, and *fyn*-overexpressing cells as N17 cells. (*top*) Anti-p120$^{c\text{-}cbl}$ immunoprecipitates from 10^7 cells were tested for phosphotyrosine. An equal amount of p120$^{c\text{-}cbl}$ was analyzed (data not shown). (*Bottom*) Whole-cell lysates from 2.5×10^5 cells were tested for the amount of p59^{fyn}. (*B*) Splenic T cells (2×10^7) from wild-type and *fyn*-deficient mice were stimulated as described in *A*. Anti-p120$^{c\text{-}cbl}$ immunoprecipitates from 2×10^7 cells were tested for phosphotyrosine. An equal amount of p120$^{c\text{-}cbl}$ was analyzed (data not shown). Positions and sizes (kD) of standard protein markers are indicated.

p55^{blk}), ZAP-70/Syk-family (p72^{syk}), and Btk/Itk/Tec-family (p77^{btk}), in a time-dependent manner (5, 23). To determine the PTKs responsible for tyrosine phosphorylation of p120$^{c\text{-}cbl}$, we used the wild-type, *lyn*-negative, and *syk*-negative DT40 chicken B cells. Wild-type DT40 cells predominantly express *lyn* and *syk* among the *src*-family and *zap*-70/*syk*-family genes, respectively. *lyn*-negative and *syk*-negative DT40 cells were established by gene targeting (17). Compared with wild-type DT40 cells, overall tyrosine phosphorylation upon BCR stimulation was reduced in *lyn*-negative and *syk*-negative DT40 cells (reference 17 and Fig. 4, *bottom panels*). As shown in Fig. 4 *A*, BCR stimulation induced rapid tyrosine phosphorylation of p120$^{c\text{-}cbl}$ in wild-type DT40 cells as in WEHI-231 cells (Fig. 2). The amount of p120$^{c\text{-}cbl}$ was unchanged during the assay period (Fig. 4, *middle panels*). In contrast, tyrosine phosphorylation of p120$^{c\text{-}cbl}$ was almost abolished in *lyn*-negative cells after BCR stimulation (Fig. 4 *B*). In *syk*-negative cells, p120$^{c\text{-}cbl}$ was tyrosine phosphorylated in a time-dependent manner

similar to that in wild-type cells, although the level was slightly lower (Fig. 4 *C*). Thus, p53/56^{lyn} was crucial for tyrosine phosphorylation of p120$^{c\text{-}cbl}$ upon activation through the BCR. In addition, the time course of p120$^{c\text{-}cbl}$ phosphorylation is similar to that of activation of p53/p56^{lyn} rather than that of p72^{syk} or p77^{btk} (2, 23), suggesting that p120$^{c\text{-}cbl}$ is a substrate of Src-family PTKs.

A low level of tyrosine phosphorylation of p120$^{c\text{-}cbl}$ was observed in *lyn*-negative DT40 cells at 3 and 10 min after BCR stimulation (Fig. 4 *B*). p72^{syk} could be activated in the absence of p53/56^{lyn}, although maximal activation of p72^{syk} required p53/56^{lyn} (24). The level of tyrosine phosphorylation of p120$^{c\text{-}cbl}$ was only slightly less in *syk*-negative DT40 cells than that in wild-type DT40 cells (Fig. 4 *C*). Thus, ZAP-70/Syk-family PTKs may contribute to a lesser extent to tyrosine phosphorylation of p120$^{c\text{-}cbl}$.

Another Src-family PTK, p59^{fyn}, plays an important role in TCR-mediated signaling (5). In addition, p120$^{c\text{-}cbl}$ was rapidly tyrosine phosphorylated upon stimulation of the TCR in Jurkat T cells (13; Tezuka, T., and T. Yamamoto, unpublished data). To further confirm the functional interaction of p120$^{c\text{-}cbl}$ with Src-family PTKs, we investigated the involvement of p59^{fyn} in tyrosine phosphorylation of p120$^{c\text{-}cbl}$ upon TCR stimulation. The *fyn*-overexpressing T cell hybridomas, N17 cells, *fyn*-deficient mice were used. Expression of p59^{fyn} was at least five times higher in N17 cells compared with that in parental HBC cells (reference 18 and Fig. 5 *A*, *bottom*). Both HBC and N17 cells exhibited increased tyrosine phosphorylation of p120$^{c\text{-}cbl}$ by CD3ε cross-linking (Fig. 5 *A*, *top*). Apparently, tyrosine phosphorylation of p120$^{c\text{-}cbl}$ was elevated in *fyn*-overexpressing cells compared with that in parental cells. To confirm the importance of p59^{fyn} in tyrosine phosphorylation of p120$^{c\text{-}cbl}$, we prepared splenic T cells from wild-type and *fyn*-deficient mice (19). Tyrosine phosphorylation of p120$^{c\text{-}cbl}$ was significantly increased upon TCR stimulation in splenic T cells from wild-type mice. In contrast, elevation of tyrosine phosphorylation of p120$^{c\text{-}cbl}$ was completely absent in splenic T cells from *fyn*-deficient mice (Fig. 5 *B*). Based on these results, we concluded that p59^{fyn} played an essential role in tyrosine phosphorylation of p120$^{c\text{-}cbl}$ upon TCR-mediated signaling.

Tyrosine phosphorylation of p120$^{c\text{-}cbl}$ has been correlated with tumorigenicity of p120$^{c\text{-}cbl}$ (12), suggesting that p120$^{c\text{-}cbl}$ is involved in regulation of cell proliferation. However, the mechanism by which p120$^{c\text{-}cbl}$ or the *v-cbl* oncogene product would induce growth of cells is not established. Role(s) of p120$^{c\text{-}cbl}$ in lymphocyte signaling is not known either. We have shown here that p120$^{c\text{-}cbl}$ was hardly tyrosine phosphorylated upon BCR stimulation in *lyn*-negative DT40 cells and upon TCR stimulation in mature splenic T cells from *fyn*-deficient mice. Mature splenic T cells from *fyn*-deficient mice showed impaired Ca^{2+} flux and depressed IL-2 production in spite of relatively normal proliferation (5). Therefore, p120$^{c\text{-}cbl}$ might be involved in the signaling toward Ca^{2+} flux and IL-2 production. Cross-linking of the BCR on *lyn*-negative DT40 cells evokes a delayed and slow Ca^{2+} mobilization, despite the normal kinetics of inositol-1,4,5-trisphosphate (IP_3) production (17). Thus, although IP_3 is the essential mediator for Ca^{2+} mobilization, an additional factor that involves p53/p56^{lyn} is thought to be required for the BCR-mediated Ca^{2+} mobilization (17). p53/p56^{lyn}-dependent tyrosine phosphorylation of p120$^{c\text{-}cbl}$ suggests that p120$^{c\text{-}cbl}$ is a candidate of such a factor regulated by p53/p56^{lyn}.

Upon antigenic stimulation, lymphocytes become activated and undergo either apoptosis or proliferation and differentiation. It is important to establish how p120$^{c\text{-}cbl}$ contributes to these cellular responses.

After we completed this study, p120$^{c\text{-}cbl}$ was shown to be tyrosine phosphorylated by a variety of stimuli that include EGF, CSF-1, Epo, GM-CSF, and FcγRI cross-linking (25–27). In vivo association of p120$^{c\text{-}cbl}$ with Src-family PTKs is observed in HL-60, HER14, P388D1, and SRD cells (26, 27).

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