

Characterization of Cbl Tyrosine Phosphorylation and a Cbl-Syk Complex in RBL-2H3 Cells

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Summary

Tyrosine phosphorylation of the Cbl protooncogene has been shown to occur after engagement of a number of different receptors on hematopoietic cells. However, the mechanisms by which these receptors induce Cbl tyrosine phosphorylation are poorly understood. Here we demonstrate that engagement of the high affinity IgE receptor (FcεR1) leads to the tyrosine phosphorylation of Cbl and analyze how this occurs. We show that at least part of FcεR1-induced Cbl tyrosine phosphorylation is mediated by the Syk tyrosine kinase, and that the Syk-dependent tyrosine phosphorylation of Cbl occurs mainly distal to the Cbl proline-rich region within the COOH-terminal 250 amino acids. Furthermore, we show by coprecipitation that Cbl is present in a complex with Syk before receptor engagement, that the proline-rich region of Cbl and a region of Syk comprised of the two SH2 domains and intradomain linker are required for formation of the complex, and that little or no tyrosine-phosphorylated Cbl is detected in complex with Syk. Overexpression of truncation mutants of Cbl capable of binding Syk has the effect of blocking tyrosine phosphorylation of endogenous Cbl. These results define a potentially important intramolecular interaction in mast cells and suggest a complex function for Cbl in intracellular signaling pathways.

The Ag and Ig receptors on hematopoietic cells, including the T cell and B cell antigen receptors and the receptors for the Fc components of IgE and IgG, share multiple structural and functional features (1–4). All consist of multiple subunits, with the extracellular ligand-binding components distinct from the signal-transducing subunits. The signaling subunits of these receptors are all thought to transduce signals primarily through tyrosine phosphorylation-mediated signaling pathways. Nonreceptor tyrosine kinases are coupled to the signaling subunits of receptors through one or more specialized motifs found in their cytoplasmic sequences, the immune receptor tyrosine-based activation motif (ITAMs)¹ (5, 6). ITAMs contain two precisely spaced tyrosines that are subject to phosphorylation by Src family kinases, resulting in the creation of binding sites for other tyrosine kinases and signaling molecules. A model that describes the initial events after receptor en-

agement has been proposed. The relevant Src kinase is rapidly activated by receptor aggregation induced by ligand or cross-linking antibody (2, 7, 8). ITAMs are rapidly tyrosine phosphorylated by the Src kinase. The Syk or ZAP-70 protein tyrosine kinase (PTK), depending on the cell type, binds to the doubly phosphorylated ITAM via tandem SH2 domains. This binding event in itself has been shown to activate the Syk kinase in vitro (9, 10). When bound to the receptor ITAMs, Syk or ZAP-70 can be tyrosine phosphorylated by the activated Src kinases. This phosphorylation mediates ZAP-70 and Syk activation (11, 12). Members of the Btk family of PTK are also regulated by receptor ligation in these systems, and have been shown to be activated by a Src kinase (13, 14). Activation of these various tyrosine kinases then leads to signal propagation via tyrosine phosphorylation of various downstream substrates.

Cbl, the product of the protooncogene *c-Cbl*, is a prominent tyrosine kinase substrate detected in these and other tyrosine kinase-mediated signaling systems (15–24). Cbl was first described as the cellular homologue of the transforming gene of the Cas-NS1 retrovirus, a murine virus capable of causing pre-B cell lymphomas and fibroblast transformation (25, 26). Examination of the Cbl primary structure reveals several apparent modular domains, includ-

¹Abbreviations used in this paper: EMEM, Eagle's MEM; hemagglutinin; HSA, human serum albumin; ITAM, immune receptor tyrosine-based activation motif; PTK, protein tyrosine kinase.

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ing an amino-terminal region found in the v-Cbl oncogene (residues 1–357), a proline-rich region (residues 481–688), a leucine zipper motif (residues 855–COOH terminus), and multiple potential SH2 binding motifs. The Cbl proline-rich region is particularly notable for containing several consensus SH3 binding motifs.

Of great importance is that the Cbl molecule has been found to bind to a number of molecules known to be critical in signal transduction. These include tyrosine kinases such as Fyn, ZAP-70, and Btk, the adapter molecules Grb2, Crk, and Nck, and the lipid/protein kinase PI3 kinase (15–18, 27–34). In addition, the product of the *Caenorhabditis elegans* SLI-1 gene, a homologue of Cbl, has been postulated to be a negative regulator of the Ras pathway based on genetic data (35). Despite these observations, the molecular function of Cbl and tyrosine-phosphorylated Cbl during tyrosine phosphorylation-mediated signaling remains unclear.

To date one critical receptor found on specialized hematopoietic cells has not yet been reported to induce the tyrosine phosphorylation of Cbl after activation. This is Fc ϵ RI, which is found on mast cells and basophils, and is responsible for binding IgE (3, 36, 37). Fc ϵ RI consists of a ligand-binding α chain, and two ITAM-containing signaling chains, β and γ , the latter of which forms a disulfide-linked homodimer. Extensive analysis reveals that the Src kinase Lyn is bound to the β chain, is activated by receptor aggregation and phosphorylates the γ chain. Phosphorylation of the γ chain ITAM enables Syk to bind and become tyrosine phosphorylated and activated. Tyrosine phosphorylation of multiple substrates ensues. The generation of recombinant vaccinia constructs encoding various cytoplasmic signaling components has simplified the analysis of tyrosine kinase signaling in this system (38). We report here that Fc ϵ RI engagement induces Cbl tyrosine phosphorylation in RBL cells. Using relevant recombinant vaccinia constructs, we identify the PTKs capable of this phosphorylation, perform preliminary mapping of the tyrosine phosphorylation of Cbl, and demonstrate a complex of Cbl and the PTK Syk. We observe that the fraction of Cbl found in complex with Syk after Fc ϵ RI triggering is far less tyrosine phosphorylated than total Cbl. These results implicate Cbl as an important participant in signaling events coupled to the Fc ϵ RI.

Materials and Methods

Cell Lines and Cell Culture. The rat mast cell line, RBL-2H3, was cultured in Eagle's MEM/16% FCS. The mouse fibroblast cell line, 3T3E, which stably expresses Fc ϵ RI α , β , and γ chains was cultured in Dulbecco's MEM/10% FCS supplemented with 300 μ g/ml of hygromycin B (Calbiochem Novabiochem, San Diego, CA) and 500 μ g/ml of G418 (Life Technologies, Gaithersburg, MD).

Antibodies. Rabbit polyclonal anti-cbl antibody C15 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-cbl antibodies, R2 and R5 (15), were reported previously. R5 antibody recognizes v-cbl (1–357 human c-cbl). Rabbit polyclonal anti-syk antibody which recognizes both rat and porcine syk was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit polyclonal anti-porcine syk

antibody and rabbit polyclonal anti-mouse lyn were reported previously (38). Anti-HA antibody, 12CA5 (mouse monoclonal), was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Rabbit polyclonal anti-GST antibody, used as a control antibody was a gift of Dr. S. Desiderio (Johns Hopkins University).

cDNAs and Recombinant Vaccinia Virus. The cDNA constructs 1-906 human hemagglutinin (HA) c-cbl (wild type) and 1-480 human HA c-cbl were reported previously (39). 1-655 human HA c-cbl was generated from pUC HUT cbl (39). 1-840 human HA c-cbl was generated by ligating synthetic oligonucleotides (5'-CTGTTAGAAGCTTTCTAGAGGTAC-3' and 3'-GAC-AATCTTCGAAAGATCTC-5') at unique PvuII site in 1-906 human HA c-cbl. All of the HA tag were added at the amino terminus. cDNAs for porcine Syk (40), porcine Syk-T (41), and murine Lyn (42) were reported previously. cDNA for kinase inactive Lyn was reported previously (13). The cDNAs were subcloned into the pSC-65 vaccinia recombination plasmid. Recombinant viruses were selected with 5-bromo-2-deoxyuridine and beta galactosidase, amplified and titered as previously described (38).

Vaccinia Infection, Cell Harvest, Cell Stimulation, and Cell Lysis. Two protocols were used for infecting and lysing RBL-2H3 cells. In one, used for experiments shown in Figs. 2, 3, and 12 cells were infected in 5% FCS for \sim 15 h in the presence of 1 μ g/ml monoclonal anti-DNP for receptor loading. Infected cells were harvested with PBS with 1 mM EDTA, washed with EMEM, and resuspended in EMEM. Cells were stimulated by adding DNP-human serum albumin (HSA) (Sigma Chemical Co., St. Louis, MO) to a final concentration of 200 ng/ml for the indicated time. Cells were lysed in 0.5% Triton X-100, 150 mM NaCl, 200 mM boric acid, pH 8.0, 5 mM EDTA, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin. For all other RBL experiments, cells were infected with 5 PFU/cell of the indicated virus in EMEM with 5% FCS for 2 h, washed once with EMEM, and incubated for 8–10 h in EMEM/5% FCS with 1 μ g/ml of monoclonal anti-DNP for surface IgE receptor loading. Infected cells were harvested with PBS with 1 mM EDTA, washed with EMEM, and resuspended in EMEM. Cells were stimulated by adding DNP-HSA (Sigma Chemical Co.) to a final concentration of 200 ng/ml for the indicated time. Cells were lysed in 1% Brij 96 lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, 1 mM sodium vanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 250 ng/ml *p*-nitrophenyl *p*'-guanidinobenzoate. 3T3E cells were plated in DMEM/10% calf serum with anti-DNP and cultured overnight. The next morning cells were infected with 5 PFU/cell in serum-free DMEM and anti-DNP (1–5 μ g/ml) again. The infection was allowed to progress for 2–4 h, with harvest and stimulation as above except 400 ng/DNP-HSA for stimulation for 4 min. Cells were lysed as described in the first RBL cell protocol.

Immunoprecipitation, Electrophoresis, and Western Blotting. Proteins in detergent lysates were immunoprecipitated, washed, and eluted by boiling in SDS sample buffer as previously described (43). Proteins were separated by SDS-PAGE, transferred to membranes, and detected by immunoblotting and enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Results

Cbl Is Tyrosine Phosphorylated after Fc ϵ RI Engagement. RBL-2H3 cells were used to determine whether Cbl would become tyrosine phosphorylated in response to Fc ϵ RI ligation.

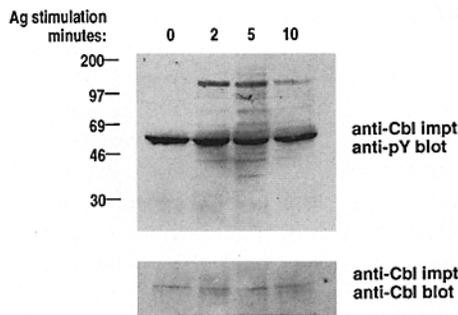


Figure 1. Antigenic stimulation via the FcεRI leads to Cbl tyrosine phosphorylation. RBL-2H3 cells were incubated for 15 h in the presence of monoclonal IgE anti-DNP. Cells were triggered or not for the indicated time with DNP-HSA and lysed. Postnuclear supernatants were subjected to SDS-PAGE, transfer, and immunoblotting with antiphosphotyrosine antibodies (*top*) or anti-Cbl antibodies (*bottom*). The prominent 50-kD band in the upper panel is Ig heavy chain.

tion. Cells were incubated overnight at 37° with an anti-DNP IgE mAb, then washed, and incubated for varying lengths of time with DNP-HSA as indicated (Fig. 1). Cbl was immunoprecipitated from detergent lysates with specific polyclonal antibodies. Immunoblotting of the electrophoresed and transferred proteins was performed with antiphosphotyrosine mAbs. No tyrosine-phosphorylated Cbl was detected in the absence of stimulation, but a prominent tyrosine-phosphorylated protein of 120-kD apparent molecular mass was detected after 2 and 5 min of stimulation. A decrease in signal was noted after 10 min of stimulation. The same membrane was stripped and subjected to reblotting with anti-Cbl antibodies indicating that the level of Cbl protein was identical in all lanes. Additional experiments (not shown) demonstrated that Cbl tyrosine phosphorylation in RBL cells could be detected as early as 30 seconds after FcεRI ligation. These studies establish that activation of the FcεRI induces tyrosine phosphorylation of Cbl in a fashion analogous to that seen with other antigen and Ig receptors.

Both Lyn and Syk Enhance FcεRI-mediated Cbl Tyrosine Phosphorylation. As a first step in analyzing how FcεRI-mediated Cbl tyrosine phosphorylation occurs, we overexpressed the Lyn and Syk tyrosine kinases to determine whether either of these protein tyrosine kinases might be responsible for tyrosine phosphorylation of Cbl in RBL-2H3 cells. Activation of the Lyn tyrosine kinase is thought to be one of the earliest events to occur after FcεRI engagement due to its involvement in phosphorylation of FcεRI ITAMs. Therefore, we first investigated the effects of Lyn overexpression on Cbl tyrosine phosphorylation. The Lyn PTK was overexpressed in RBL-2H3 cells by infection with a Lyn vaccinia recombinant virus. In parallel, cells were infected with viral vectors expressing no kinase or a kinase-inactive form of Lyn. Expression of active and inactive Lyn was detected by immunoblotting with anti-Lyn antibodies (Fig. 2, *bottom*). The cells were incubated with anti-IgE and then with or without DNP-HSA for ac-

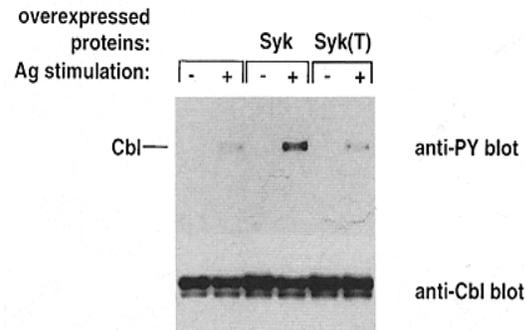


Figure 2. Overexpression of Lyn or an inactive form of Lyn in RBL-2H3 cells. RBL-2H3 cells were infected with vaccinia vector, with Lyn recombinant virus, or with a recombinant virus made with a kinase-dead form of Lyn. Cells were incubated with monoclonal IgE anti-DNP. FcεRI was aggregated (+) or not (-) with DNP-HSA. Cellular lysates were subjected to immunoprecipitation with anti-Lyn antibodies, SDS-PAGE, and immunoblotting with antiphosphotyrosine antibodies (*top*) or anti-Cbl antibodies (*middle*). For the lower panel, cellular lysates were subjected to SDS-PAGE and blotted with anti-Lyn antibodies.

tivation. FcεRI-induced Cbl tyrosine phosphorylation is not affected by the vaccinia infection (data not shown). Overexpression of an inactive form of Lyn did not affect Cbl tyrosine phosphorylation relative to the control virus, which expressed no protein. However, overexpression of wild-type Lyn resulted in an increase in the basal level of Cbl tyrosine phosphorylation and a marked increase in the level of tyrosine phosphorylation observed after FcεRI engagement. This result indicates that tyrosine phosphorylation of Cbl can be regulated by the Lyn PTK. However, because of the proximal position of Lyn in FcεRI signaling pathways, one can not determine from this experiment whether this is a direct or indirect effect. Syk activation closely follows and is dependent on activation of the Lyn PTK in this system (36). To test the possible role of Syk in Cbl phosphorylation in RBL-2H3 cells, vaccinia recombinants were used to overexpress Syk or a truncated form of Syk which lacks enzymatic activity (Syk-T) (Fig. 3). Syk-T

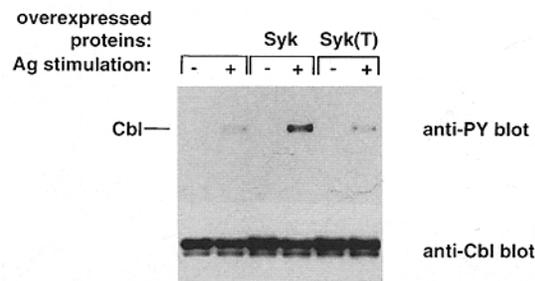


Figure 3. Overexpression of Syk or a truncated form of Syk in RBL-2H3 cells. RBL-2H3 cells were infected with vaccinia vector, with Syk recombinant virus, or with a recombinant virus made with a truncated form of Syk. Cells were incubated with monoclonal IgE anti-DNP. FcεRI was aggregated (+) or not (-) with DNP-HSA. Cellular lysates were subjected to immunoprecipitation with anti-Cbl antibodies and SDS-PAGE, and were immunoblotted with antiphosphotyrosine antibodies (*top*) or anti-Cbl (*bottom*).

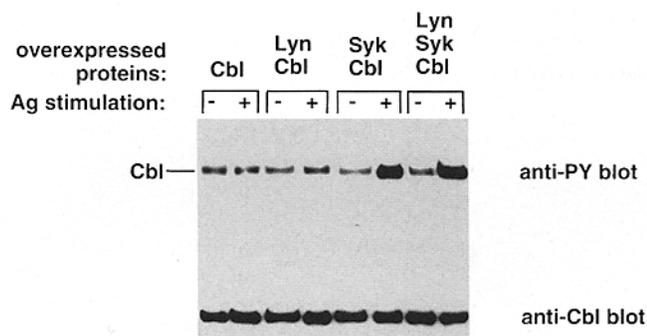


Figure 4. Overexpression of Cbl with and without Lyn and Syk or a truncated form of Syk in 3T3 cells. Cells were infected with recombinant Cbl virus, with both Cbl and Lyn recombinants, with both Cbl and Syk recombinants, or with Cbl, Lyn, and Syk recombinants. Cells were incubated with monoclonal IgE anti-DNP. Fc ϵ RI was aggregated (+) or not (-) with DNP-HSA. Cellular lysates were subjected to immunoprecipitation with anti-Cbl antibodies and SDS-PAGE, and were immunoblotted with antiphosphotyrosine antibodies (*top*) or anti-Cbl (*bottom*).

had no effect on the level of basal or receptor-induced tyrosine phosphorylation of Cbl relative to infection with the control virus. However, overexpression of wild-type Syk resulted in a severalfold increase in the level of tyrosine-phosphorylated Cbl detected after Fc ϵ RI ligation. Levels of Syk and truncated Syk were comparable (not shown). Therefore, Syk also lies in the pathway leading to Cbl tyrosine phosphorylation.

Activation of Syk Can Reconstitute Cbl Tyrosine Phosphorylation in a Nonhematopoietic Cell Line. Taken together, the above results are consistent with a mechanism for Cbl phosphorylation in which Syk directly phosphorylates Cbl, with the effect of Lyn being mediated indirectly through enhanced activation of Syk. However, RBL-2H3 cells contain other hematopoietic-specific tyrosine kinases and signaling molecules, which confound the drawing of definitive conclusions from the above experiments. To determine whether Syk was capable of directly tyrosine phosphorylating Cbl, a previously described reconstitution system was used (38, 44). In this system, NIH 3T3 cells have been stably transfected with the three Fc ϵ RI components (3T3E cells). Since NIH-3T3 cells lack all known hematopoietic

specific tyrosine kinases, only very weak Fc ϵ RI phosphorylation occurs in the absence of exogenous kinase expression in 3T3E cells. When the Lyn kinase is expressed in them, Fc ϵ RI clustering leads to a normal level of phosphorylation. In addition, Syk expression alone allows weak but detectable activation of Syk via the receptor, while expression of both Lyn and Syk results in robust Syk activation similar to that found in RBL-2H3 cells. These events have now been shown to almost exactly reproduce those which occur in natural Fc ϵ RI signaling systems (44). To analyze how Lyn and Syk were contributing to Cbl tyrosine phosphorylation, 3T3E cells were infected with a recombinant vaccinia-Cbl virus alone, or with Cbl plus one, or both of the PTKs (Fig. 4). A basal level of Cbl tyrosine phosphorylation was detected in the presence of the endogenous PTKs in these cells. There was no increase in tyrosine phosphorylation detected after receptor ligation in the absence of either Lyn or Syk. Infection with Lyn resulted in no increase in the basal or stimulated level of Cbl tyrosine phosphorylation. However, expression of Syk followed by receptor ligation led to a marked increase in Cbl tyrosine phosphorylation. The addition of Lyn with Syk did not result in a greater level of tyrosine phosphorylation than when Syk alone was expressed with Cbl. These results indicate that in the absence of the Syk PTK, Cbl is not tyrosine phosphorylated by Fc ϵ RI ligation. In addition, since other hematopoietic-specific tyrosine kinases, such as those of the Btk family, are absent, these data strongly suggest that activated Syk is able to directly tyrosine phosphorylate Cbl.

Syk-dependent Cbl Tyrosine Phosphorylation Occurs Distal to the Cbl Proline-Rich Region. The next series of experiments was undertaken to further explore the function of Syk in the tyrosine phosphorylation of Cbl in RBL-2H3 cells. We first endeavored to set up experimental conditions in which Syk-dependent phosphorylation of Cbl could be readily assayed. Therefore, we analyzed Cbl tyrosine phosphorylation induced in RBL-2H3 cells after overexpression of Cbl and Syk or truncated Syk (Fig. 5). As was seen in the earlier experiments where Cbl was not overexpressed, there was a rapid and marked increase in the tyrosine phosphorylation of Cbl dependent on overexpression of full-length Syk. Tyrosine phosphorylation detected by immunoblotting was

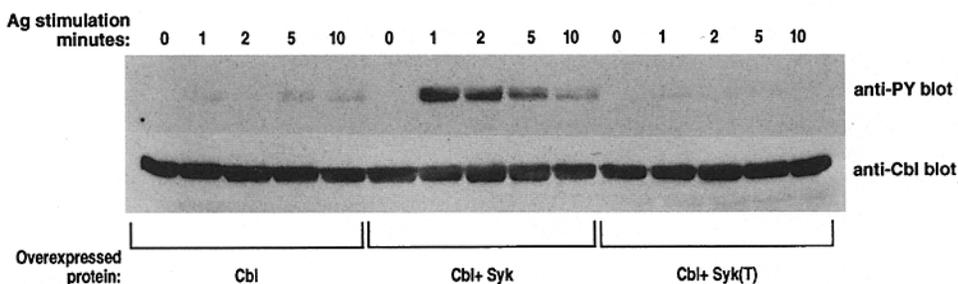


Figure 5. Time course of Cbl tyrosine phosphorylation in RBL-2H3 cells overexpressing Cbl alone or with active and inactive forms of Syk. RBL-2H3 cells were infected with recombinant Cbl virus plus vector or with either wild-type or truncated Syk recombinant virus. Cells were incubated with monoclonal IgE anti-DNP. Fc ϵ RI was aggregated for the indicated amount of time with DNP-HSA. Cellular lysates were subjected to immunoprecipitation with anti-Cbl antibodies and SDS-PAGE, and were immunoblotted with antiphosphotyrosine antibodies (*top*) or anti-Cbl (*bottom*).

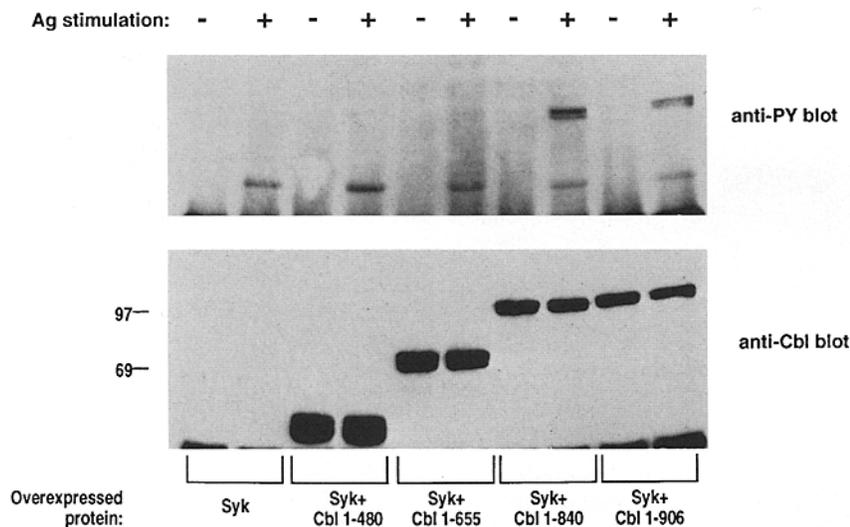


Figure 6. Truncation mapping of Cbl tyrosine phosphorylation after Syk overexpression. RBL-2H3 cells were infected with recombinant Syk virus plus vector or with truncated or wild-type Cbl recombinant virus. Cells were incubated with monoclonal IgE anti-DNP. FcεRI was aggregated (+) or not (-) with DNP-HSA. Cellular lysates were subjected to immunoprecipitation with monoclonal anti-HA antibodies. Immunoprecipitates were resolved on SDS-PAGE and immunoblotted with antiphosphotyrosine antibodies (*top*) or R5 anti-Cbl (*bottom*).

maximal by 1 min of stimulation. A gradual decrease in tyrosine-phosphorylated Cbl was detected over 10 min. There was no increase in phosphorylation with overexpression of the truncated (inactive) form of Syk. The overexpression of Syk in these conditions makes Syk the most prominent tyrosine phosphoprotein detectable in cell lysates (Scharenberg, A.M., and J.-P. Kinet, unpublished data), and these experimental conditions therefore provide a system in which Cbl tyrosine phosphorylation is dependent on Syk rather than other tyrosine kinases.

To map the site of Cbl tyrosine phosphorylation, a series of truncation mutations of Cbl were constructed with HA epitope tags at the NH₂ terminus. These were then expressed as vaccinia recombinants for expression in RBL-2H3 cells. Truncation of the COOH terminus of the full-length 906 amino acid protein to create an 840 residue truncated protein removes a leucine-rich region. Generation of a 655 amino acid construct removes an acidic region. Cbl 1-480 lacks a proline-rich region, which is postulated to be involved in protein-protein interactions mediated by SH3-containing proteins. All constructs were expressed and immunoprecipitated using anti-HA antibodies. Immunoblotting with anti-Cbl antibodies, which detect the different truncated forms of Cbl, revealed comparable levels of protein (Fig. 6). Syk was expressed alone or with the different Cbl constructs. Tyrosine phosphorylation of overexpressed, full-length Cbl is clearly detected. Similarly, tyrosine phosphorylation of Cbl 1-840 is present after FcεRI ligation. This truncation results in a loss of two tyrosine residues. Further truncation of Cbl to 655 amino acids results in marked, if not complete, loss of tyrosine phosphorylation. Five additional tyrosine residues are removed in this deletion. We detect no tyrosine phosphorylation of the NH₂-terminal 480 residues of Cbl. The results suggest that the predominant sites of Syk-dependent tyrosine phosphorylation are in the COOH-terminal 250 amino acids.

Syk Exists in a Complex with Cbl in Both Unstimulated and Stimulated Cells. The ability of Syk to tyrosine phosphorylate Cbl even in nonhematopoietic cells strongly suggests that Cbl and Syk might interact directly. In addition, as discussed above, there is ample precedent for interactions of Cbl with critical signaling proteins. We thus asked whether a complex of Cbl and Syk could be demonstrated. To address such a possibility, activated and nonactivated RBL-2H3 cells were lysed and subjected to immunoprecipitation with antibodies directed at Cbl and Syk, and, as a control, with irrelevant antibodies (Fig. 7). Immunoblotting with anti-Cbl and control antibodies confirmed that the anti-Cbl immunoprecipitations were specific. More importantly, precipitation with anti-Syk antibodies followed by probing with anti-Cbl reagents revealed that a Cbl-Syk

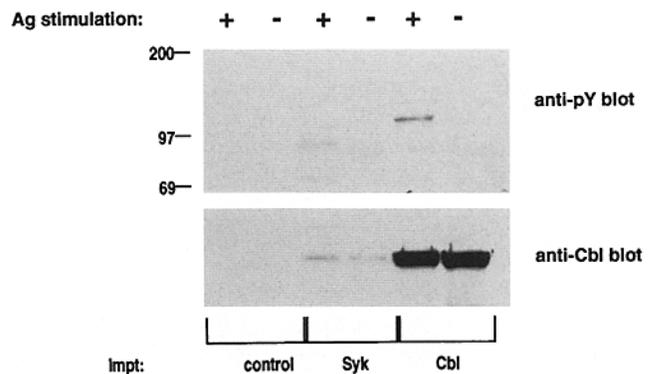


Figure 7. A Cbl-Syk complex is found without protein overexpression. RBL-2H3 cells were incubated for 15 h in the presence of monoclonal IgE anti-DNP. Cells were triggered or not for the indicated time with DNP-HSA and lysed. Postnuclear supernatants were subjected to immunoprecipitation with control antibodies or antibodies directed at Syk or Cbl. Eluted protein was subjected to SDS-PAGE, transfer, and immunoblotting with antiphosphotyrosine antibodies (*top*) or anti-Cbl antibodies (*bottom*).

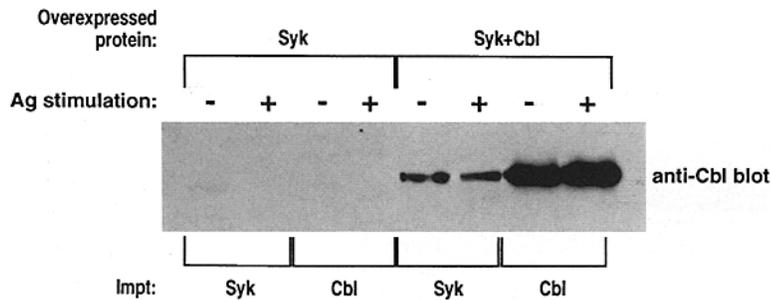


Figure 8. Identification of a Cbl-Syk complex. RBL-2H3 cells were infected with recombinant Syk virus plus vector or with both recombinant Syk and Cbl viruses. Cells were incubated with monoclonal IgE anti-DNP. FcεRI was aggregated (+) or not (-) with DNP-HSA. Cellular lysates were subjected to immunoprecipitation with the antibodies directed against either Syk or Cbl. Proteins were electrophoresed and immunoblotted with anti-Cbl antibodies. The film shown in this experiment was exposed for 5 s.

complex could be found. No differences in the amount of this complex could be detected after activation of the cells via the FcεRI.

To further analyze and map the Cbl-Syk interaction, coprecipitation conditions after overexpression of Syk and Cbl were defined in RBL-2H3 cells (Fig. 8). Syk was overexpressed with or without Cbl. Without overexpression of Cbl, the brief exposure of the film (5 s) was insufficient to demonstrate endogenous Cbl with anti-Cbl antibodies, or the complex of Syk with endogenous Cbl using anti-Syk antibodies. Cbl and the Cbl-Syk complex were detected in longer exposures. The overexpression of Cbl was clearly demonstrated by anti-Cbl immunoprecipitation and immunoblotting. Antibodies to Syk coprecipitated a large amount of the overexpressed Cbl protein. No Cbl was detected when irrelevant antibodies were used in control immunoprecipitations. There was no change in the amount of Cbl coprecipitation after activation of the cells. Therefore, these conditions accurately represent the Cbl/Syk interaction found in the absence of overexpression.

The Cbl truncation mutants were then used to define the portion of the molecule involved in the interaction with Syk. Syk was overexpressed with the full-length and truncated forms of Cbl (Fig. 9). A complex of Syk with full-length Cbl was detected. Truncations of Cbl contain-

ing amino acids 1-840 or 1-655 also bound to Syk. However, expression of a Cbl truncation generated by removal of the proline-rich domain (Cbl 1-480) failed to form a complex with Syk. Additional experiments (not shown) confirmed that the Cbl 1-480 truncation can be detected above the heavy chain band of immunoglobulin. Another experiment was performed to test whether truncation of Syk abrogates interaction with Cbl (Fig. 10). Anti-Syk immunoprecipitates were subjected to electrophoresis and immunoblotting with anti-Cbl antibodies. This experiment revealed that both full-length and truncated Syk binds to the Cbl protein. A similar experiment demonstrates that the truncated Syk protein binds truncated Cbl (1-655) (not shown).

Cbl Found in Complex with Syk in Activated Cells Demonstrates Diminished Tyrosine Phosphorylation. During the coprecipitation experiments, it was noted that tyrosine-phosphorylated Cbl was rarely detected in anti-Syk immunoprecipitates. This is demonstrated in an overexpression study in which RBL-2H3 cells were infected with both Cbl and Syk and activated or not via FcεRI (Fig. 11). The cells were then lysed, and antibodies to Syk and Cbl, as well as control antibodies, were used for immunoprecipitation. Control immunoprecipitations demonstrated that the Cbl coprecipitation with Syk is specific. A tyrosine-phosphorylated protein likely to be Syk appears to coprecipitate with Cbl (*last lane*). Of note is that the Cbl directly precipitated from lysates of activated cells is heavily tyrosine phosphorylated. In contrast, the Cbl that coprecipitates with Syk from these same

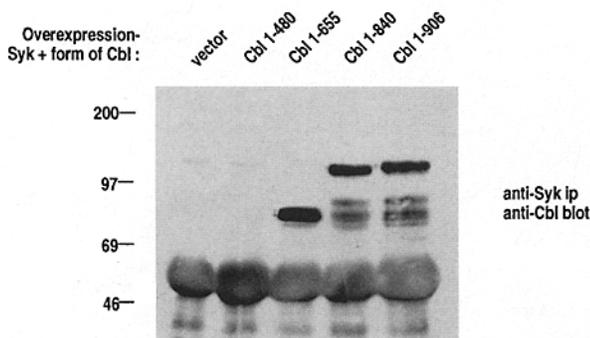


Figure 9. Syk binds truncated Cbl. RBL-2H3 cells were infected with recombinant Syk virus plus vector or with truncated or wild-type Cbl recombinant virus. Cellular lysates were subjected to immunoprecipitation with antibodies directed against Syk. Proteins were electrophoresed and immunoblotted with R5 anti-Cbl antibodies. The film was exposed for 5 s. In a 2-min exposure, the association between Syk and endogenous Cbl was detected in cells without Cbl overexpression.

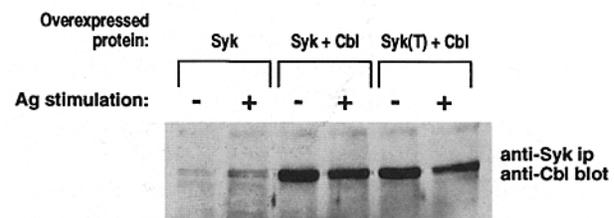


Figure 10. Cbl binds truncated Syk. RBL-2H3 cells were infected with recombinant Syk virus plus vector, recombinant Syk and Cbl recombinant viruses, or with truncated Syk and Cbl viruses. Cellular lysates were subjected to immunoprecipitation with the antibodies directed against Syk. Proteins were electrophoresed and immunoblotted with anti-Cbl antibodies. Exposure of the film was for 1 min. A 5-s exposure was not sufficient to detect endogenous Cbl in lane 2.

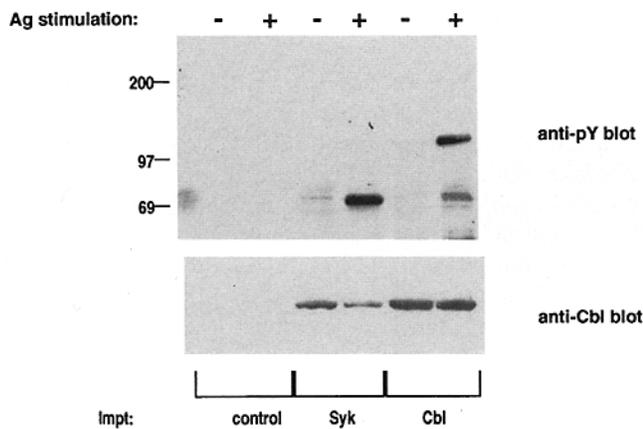


Figure 11. Cbl in complex with Syk is not tyrosine phosphorylated. RBL-2H3 cells were infected with recombinant Syk and Cbl viruses. Cells were incubated with monoclonal IgE anti-DNP. Fc ϵ RI was aggregated (+) or not (-) with DNP-HSA. Cellular lysates were subjected to immunoprecipitation with control antibodies or antibodies directed against either Syk or Cbl. Proteins were electrophoresed and immunoblotted with antiphosphotyrosine (*top*) or anti-Cbl (*bottom*) antibodies.

lysates contains markedly less tyrosine phosphate. This is despite the presence of comparable levels of Cbl in these two precipitates as detected by anti-Cbl blotting.

Overexpression of Cbl Truncation Mutants Capable of Binding Syk Results in Inhibition of Endogenous Cbl Tyrosine Phosphorylation. The absence of tyrosine-phosphorylated Cbl within the Cbl-Syk complex suggested that a function of this complex might be to regulate Cbl tyrosine phosphorylation. The ability to selectively isolate endogenous Cbl protein away from the overexpressed form of the protein enabled us to test the effect of Cbl overexpression on the endogenous form of the protein. Specifically, we could ask whether overexpression of Cbl led to changes in tyrosine

phosphorylation of endogenous Cbl. In this experiment (Fig. 12) RBL-2H3 cells were separately infected with vector or with recombinant vaccinia encoding either of three truncation mutants of Cbl, 1-480, 1-655, or 1-840. The three forms were well expressed as detected by blotting with antibodies to the NH₂-terminal HA tag (*bottom*). Endogenous Cbl was detected by immunoprecipitation and immunoblotting with an antibody directed against the COOH-terminal 15 amino acids of the protein and therefore not found in the three truncation mutants (*middle*). Stimulation of the cells by cross-linking the Fc ϵ RI receptor leads to an increase in tyrosine phosphorylation of endogenous Cbl in cells infected with vector alone. Overexpression of the 1-480 Cbl truncation mutant had no effect on the tyrosine phosphorylation of endogenous Cbl induced by receptor ligation. However, there was a marked inhibition of this tyrosine phosphorylation with overexpression of the 1-655 or 1-840 mutants. Note that these are two truncation mutants shown earlier (Fig. 9) to bind to Syk. Thus overexpression of Cbl truncation mutants that are capable of binding Syk has the functional effect of blocking tyrosine phosphorylation of endogenous Cbl.

Discussion

It has been previously established that the Cbl protein is a prominent tyrosine kinase substrate in a number of signaling pathways, including those of several antigen receptors and a variety of growth factor and cytokine receptors (15-24, 29). The apparent ubiquitous nature of Cbl involvement in tyrosine kinase signaling pathways suggests that Cbl plays a general and critical role in tyrosine kinase-mediated signal transduction. However, at present there is no clear function which can be attributed to Cbl or tyrosine-phosphorylated Cbl. Understanding how Cbl tyrosine phosphorylation is generated is a necessary first step in under-

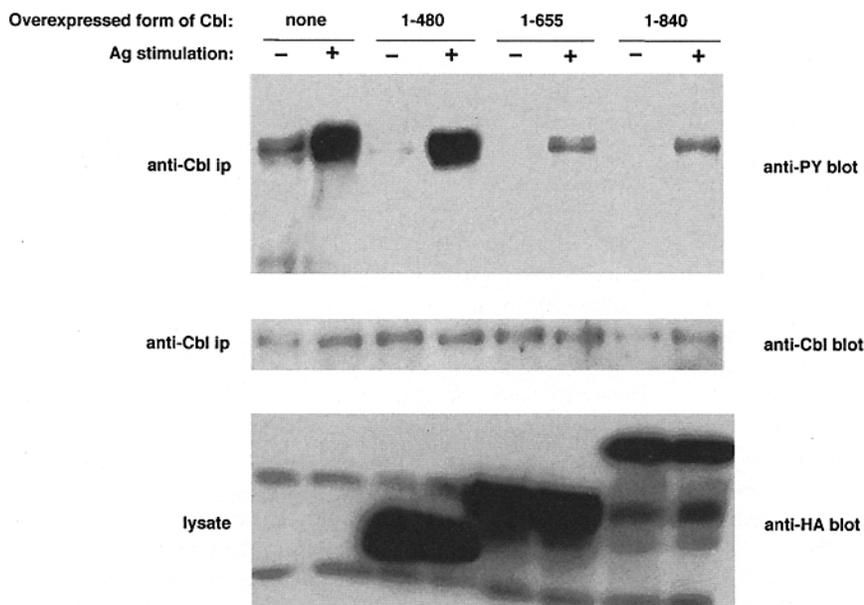


Figure 12. Overexpression of truncation mutants of Cbl inhibits tyrosine phosphorylation of endogenous Cbl. RBL-2H3 cells were infected with vector or one of three Cbl truncation mutants (1-480, 1-655, or 1-840) expressed as recombinant vaccinia viruses. Fc ϵ RI was aggregated (+) or not (-) with DNP-HSA. Cellular lysates were directly electrophoresed (*bottom*) or were subjected to immunoprecipitation with antibodies directed against the carboxy terminus of Cbl before electrophoresis. Proteins were immunoblotted with antiphosphotyrosine (*top*), anti-Cbl (*middle*), or anti-HA antibodies (*bottom*).

standing Cbl function. We have performed a detailed analysis of FcεRI-mediated tyrosine phosphorylation of Cbl using a vaccinia virus-mediated expression approach.

In our studies, overexpression of either Lyn or Syk PTKs in RBL-2H3 cells resulted in enhanced Cbl tyrosine phosphorylation. A caveat for these experiments is that one cannot be sure that overexpression of a particular PTK and enhanced subsequent tyrosine phosphorylation of a substrate indicate that the overexpressed kinase is directly responsible for the phosphorylation, as the possibility that one PTK can activate another is well known in these systems. Therefore, these experiments suggest that either Lyn or Syk, or a PTK that is downstream to them, is responsible for Cbl tyrosine phosphorylation. However, the complementary reconstitution analysis in the nonhematopoietic 3T3 cell line provides strong evidence that Syk is able to directly tyrosine phosphorylate Cbl. The requirement for Src kinase activation of Syk was not addressed in these experiments, but in view of previous studies, it is possible that endogenous Src kinases might have contributed to activation of Syk either in the RBL-2H3 or 3T3 cells. The conclusion that Syk is primarily responsible for Cbl tyrosine phosphorylation needs to be considered in the context of other studies. In B cell receptor-mediated signaling, Tezuka et al. (45) demonstrated that Cbl tyrosine phosphorylation occurred in DT40 chicken cells upon IgM cross-linking. This phosphorylation was almost fully abrogated in Lyn-negative cells, but persisted in Syk-negative DT40 cells. In related experiments they found that overexpression of Fyn in a T cell hybridoma resulted in enhanced Cbl tyrosine phosphorylation. Blockade of ZAP-70 activation either with a non-hydrolyzable analogue of a TCR-ζ ITAM or by overexpression of the tandem SH2 domains of ZAP-70 resulted in complete inhibition of the tyrosine phosphorylation of a 36-kD substrate, but only partial inhibition of the phosphorylation of the 120-kD protein likely to be Cbl (46, 47). It is thus likely that Cbl is a substrate for both Src and Syk family PTKs with the relative contribution varying in the different cellular systems.

The function of Cbl tyrosine phosphorylation has not yet been fully determined. The preliminary truncation mapping presented in this study suggests that tyrosine residues distal to amino acid 655 are likely to be the major target of activated Syk in RBL-2H3 cells. However, the conditions of overexpression used in these studies emphasize Syk dependent phosphorylation of Cbl, and therefore leave open the possibility that proximal sites of Cbl phosphorylation occur and may be mediated by other tyrosine kinases. Several recent studies indicate that upon its tyrosine phosphorylation Cbl can be bound by glutathione-S-transferase-SH2 fusion proteins *in vitro* (15, 29). Recently published data suggest that Cbl interaction with Crk proteins is mediated by the Crk SH2 domain (48). Inspection of the Cbl sequence indicates a number of tyrosine residues, which when tyrosine phosphorylated, are in the appropriate context to bind various SH2 domains. These studies strongly suggest that tyrosine phosphorylation of Cbl is in-

timately involved in determining protein-protein interactions.

Our data further indicate that in RBL-2H3 cells, Syk and Cbl exist in a preformed complex. The binding data using Cbl and Syk truncation mutants, suggest that binding of Syk to Cbl is mediated through the proline-rich region of Cbl. In addition, truncation of the Syk protein to leave the tandem SH2 domains and a short linker region fails to inhibit binding to Cbl. The ability of this portion of Syk to bind Cbl in the absence of cell activation may be related to a number of recent observations of phosphotyrosine-independent binding of SH2 domains. The SH2 domain of Abl binding to Bcr (49), Src and Fyn SH2 binding to Raf (50), Blk SH2 binding to a novel 130-kD protein (51) and Lck SH2 binding to a novel 62-kD protein (52) are potential precedents to the observations we report. Alternatively the site of interaction with Cbl may prove to be the intra-SH2 domain linker region.

Cbl is now well documented to interact with a number of critical tyrosine kinases. Association with Src family kinases has been shown in T and B cells (29, 53). However, in contrast to our results, Panchamoorthy et al. (53) showed that a Cbl-Syk complex was induced by B cell receptor ligation in Ramos B cells. We do not observe changes in the level of protein association in normal RBL-2H3 cells or reproducibly after protein overexpression. The differences between our results and these other studies may be due to differences in such experimental conditions as immunological reagents.

The absence of tyrosine phosphorylation of the Cbl within the Cbl-Syk complexes we observe in RBL-2H3 cells is surprising. In two previous studies reporting interactions between Cbl and Syk or ZAP-70 kinases, the Cbl coprecipitating with Syk or ZAP-70 was shown to be tyrosine phosphorylated. The differences between our results and these studies may again be due to differences in immunological reagents, or possibly because the experimental conditions in our studies emphasize Syk-dependent phosphorylation of Cbl over that mediated by other tyrosine kinases. The data supporting the conclusion that Syk is involved in this tyrosine phosphorylation and the apparent direct physical interaction between Syk and Cbl led us to expect that that fraction of Cbl binding Syk might be the fraction of Cbl subject to phosphorylation. However, it is possible that the complex might represent an interaction that leads to phosphorylation or alternatively, an interaction that leads to regulation of Syk activity.

An additional clue about the function of the Cbl-Syk complex comes from an experiment in which truncation mutants of Cbl were overexpressed in RBL-2H3 cells. Overexpression of those mutants that were shown to bind Syk, 1-655, and 1-840, were able to inhibit tyrosine phosphorylation of endogenous Cbl. A truncation mutant that fails to bind Syk fails to inhibit tyrosine phosphorylation. At this point there are several potential explanations for a result that can be viewed as the dominant negative effect of mutant overexpression. The mutant forms of Cbl might be

displacing endogenous Cbl from Syk thereby preventing necessary contact with the tyrosine kinase molecule that would normally phosphorylate it. Alternatively, overexpression of Syk-binding truncation mutants might lead to Syk inhibition. These are essentially the same explanations provided above for the finding that Cbl in complex with Syk is not tyrosine phosphorylated. Both results emphasize how tightly tyrosine phosphorylation of Cbl is regulated, and demonstrate that a function of Cbl in this system is to form complexes with critical tyrosine kinases. Whether Cbl in these complexes functions to regulate kinase activity and cellular function is currently under study.

The *C. elegans* Cbl homologue has been postulated to be a negative regulator of the Ras pathway (35). Cbl has been

shown to act as an adapter molecule in coupling epidermal growth factor receptor to PI-3 kinase (20, 21). Another study has suggested that Cbl targets ubiquitination and degradation of the CSF-1 receptor (54). These functions may be mediated by the interactions of SH3-containing proteins with the proline-rich domain of Cbl or by SH2-containing proteins interacting with tyrosine-phosphorylated Cbl. Our observations indicating an interaction between the SH2 domain/linker regions of Syk and non-tyrosine-phosphorylated Cbl suggests that additional modes of interaction with Cbl may exist. Further characterization of this Cbl-Syk complex and evaluation of Syk kinase function when complexed with Cbl may lead to more insight into the role of this complex protein.

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