The RING Finger Domain of Cbl Is Essential for Negative Regulation of the Syk Tyrosine Kinase*

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The proto-oncogene product Cbl has emerged as a negative regulator of a number of protein-tyrosine kinases, including the ZAP-70/Syk tyrosine kinases that are critical for signaling in hematopoietic cells. The evolutionarily conserved N-terminal tyrosine kinase-binding domain is required for Cbl to associate with ZAP-70/Syk and for their subsequent negative regulation. However, the role of the remaining C-terminal regions of Cbl remains unclear. Here, we used a COS-7 cell reconstitution system to address this question. Analysis of a series of C-terminally truncated Cbl mutants revealed that the N-terminal half of the protein, including the TKB and RING finger domains, was sufficient to mediate negative regulation of Syk. Further truncations, which delete the RING finger domain, abrogated the negative regulatory effects of Cbl on Syk. Point mutations of conserved cysteine residues or a histidine in the RING finger domain, which are required for zinc binding, abrogated the ability of Cbl to negatively regulate Syk in COS-7 cells and Ramos B lymphocytic cells. In addition, Syk-dependent transactivation of a serum response element-luciferase reporter in transfected 293T cells was reduced by wild type Cbl; mutations of the RING finger domain or its deletion abrogated this effect. These results establish the RING finger domain as an essential element in Cbl-mediated negative regulation of a tyrosine kinase and reveal that the evolutionarily conserved N-terminal half of the protein is sufficient for this function.

The protein product of the proto-oncogene c-cbl has emerged as a prominent component of protein-tyrosine kinase (PTK) mediated signaling cascades downstream of the activated cell surface receptors. These receptors include the T cell receptor (TCR), the B cell receptor, Fc receptors, and cytokine receptors that activate non-receptor PTKs (1–6). Cbl also functions downstream of a number of receptor PTKs, such as the platelet-derived growth factor receptor (PDGFR) and the epidermal growth factor receptor (EGFR). The N-terminal region of Cbl (Cbl-N; aa 1–357), corresponding to sequences retained in the v-cbl oncogene, functions as a tyrosine kinase-binding (TKB) domain that interacts directly with autophosphorylated PTKs, including the lymphocyte antigen receptor-associated Syk and ZAP-70 PTKs, and receptor PTKs PDGFR α/β and EGFR (7–14). The recently solved crystal structure has demonstrated that the Cbl TKB domain is composed of a four-helical bundle, a calcium-binding EF hand, and a variant SH2 domain (15) (Fig. 1A). The SH2 domain lacks certain critical loop structures and is unable to bind to phosphotyrosine-containing peptides on its own. The three constituent domains cooperatively create a novel phosphopeptide-binding platform.

The Cbl polypeptide also contains a numbers of other domains or motifs as follows: a RING finger domain whose function has not been determined; an extensive proline-rich region (amino acids 481–690) that provides binding sites for the SH3 domain of Src family PTKs and the adapter proteins Grb2 and Nck (1–6); and a C-terminal leucine zipper that has significant homology to ubiquitin-associated domains found in ubiquitin machinery enzymes and certain targets (16, 17). Receptor-induced phosphorylation of specific tyrosine residues in C-terminal half of the Cbl protein creates docking sites for the SH2 domain-containing proteins, such as VAV (Tyr-700), the p85 subunit of phosphatidylinositol-3 kinase (Tyr-731), and the adapter proteins of the Crk family (Tyr-774) (18–20).

Recently, two additional mammalian Cbl-related genes have been cloned. Cbl-b is highly related to Cbl in its primary structure and the arrangement of its domains (1, 21). In contrast, Cbl-SL (for Cbl-SLI-1-like)/Cbl-3 is more distantly related to Cbl and Cbl-b, and its domain structure closely resembles that of the Caenorhabditis elegans Cbl homologue SLI-1 (6, 22). Both Cbl-SL/Cbl-3 and SLI-1 possess the TKB and RING finger domains and a very short proline-rich region but lack the remaining C-terminal sequences including the leucine zipper. The recently cloned Drosophila Cbl (D-Cbl) contains only the TKB and RING finger domains (23, 24). A comparison of all the known Cbl family members emphasizes the conservation of the TKB and RING finger domain sequences, suggesting that these domains may be important for an evolutionarily conserved role of this family of proteins (6).

Recent genetic and biochemical studies have revealed an evolutionarily conserved role of the Cbl family proteins as negative regulators of tyrosine kinase signaling cascades (1–6,
Various cell types was shown to down-regulate the EGFR and example, the overexpression of exogenous wild type Cbl in point to a direct effect of Cbl at the level of PTKs. For role of Cbl for PTK signaling pathways and, importantly, have experiments also have demonstrated the negative regulatory tion, endocytosis, and degradation (28, 30–32).

To involve an enhancement of their ligand-induced ubiquitina- tion, endocytosis, and degradation (28, 30–32).

Overexpression of Cbl in the RBL-2H3 mast cell line was shown to reduce the autophosphorylation and kinase activity of co-expressed Syk and inhibit histamine release following FceRI ligation (33). Notably, the TKB domain of Cbl binds directly to the negative regulatory phosphorylation site within the SH2 kinase linker region of ZAP-70 (Tyr-292) and Syk (Tyr-323) PTKs (11–13). The Tyr → Phe mutations of these sites render ZAP-70 and Syk hyperactive in vivo when expressed in lymphoid cells, whereas the in vitro kinase activity of ZAP-70-Y292F was unchanged (34–36). Similar experiments in a COS-7 cell system demonstrated that co-expression of Cbl induced a loss of the kinase-active pool of Syk, and this effect was accompanied by a decrease in the level of Syk protein (12). These studies have clearly established a critical role for Cbl in the negative regulation of Syk/ZAP-70 PTKs.

Importantly, a TKB domain-inactivating mutation (G306E), corresponding to a loss of function mutation in SLI-1, blocked the binding of Cbl to Syk and ZAP-70 PTKs and abrogated the Cbl-dependent negative regulatory effect on Syk (11, 12). Conversely, Syk Y323F mutant was resistant to Cbl-induced negative regulation (12). Recent studies have demonstrated a similar requirement for the TKB domain in Cbl-mediated negative regulation of receptor PTKs (28, 32). However, given the dominant oncogenic activity of the TKB domain and its ability to up-regulate PTK signaling, it is clear that additional C-terminal sequences in Cbl are required for its negative regulatory effect on PTKs.

In this study, we have used transfection studies in COS-7, 293T, and Ramos B lymphocytic cells to assess the role of these additional sequences in Cbl-dependent negative regulation of the Syk PTK. Our results demonstrate that the RING finger domain of Cbl is essential for the negative regulation of Syk. Furthermore, the N-terminal portion of Cbl, which include the TKB and RING finger domains, is sufficient for this function. The evolutionary conservation of the Cbl domains that are required for negative regulation of Syk suggests that these findings should be generalizable to Cbl-dependent negative regulation of other PTKs.

EXPERIMENTAL PROCEDURES

Cells—COS-7 and 293T cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s media supplemented with 10% fetal calf serum (HyClone), 20 mM HEPES, pH 7.35, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Life Technologies, Inc.). Ramos-T cell line was established by electroporating the human surface IgG1-Fly photolytic cell line Ramos with the pCMVneo.SVT vector (SV40 T antigen DNA sequences cloned in the pCMVneo vector downstream of the CMV promoter, a gift from Dr. Vinm Band, New England Medical Center, Boston). High SV40 T antigen expression was verified by immunoprecipitation data (not shown). Ramos-T cell line was maintained as described for Ramos cells (37) with the addition of 500 µg/ml G418.

Expression Plasmids and Site-directed Mutagenesis—The pSR Neo plasmid, encoding the extracellular and trans-membrane domains of human CDS fused to the cytoplasmic tail of human TCR γ chain, has been described (11). All Cbl expression constructs use the pSR-Neo plasmid backbone with a CMV promoter for mammalian expression (Promega Corp., Madison, WI). The wild type or mutant Cbl proteins were designed to include an N-terminal HA tag. The pAlterMAX-HA-Cbl, encoding the wild type Cbl, and pAlterMAX-HA-Cbl-G306E encoding its G306E mutant, have been described (11). The pAlterMAX constructs encoding the HA-tagged truncation mutants, Cbl-655 (aa 1–655), Cbl-480 (aa 1–480), Cbl-436 (aa 1–436), Cbl-421 (aa 1–421), and Cbl-357 (aa 1–357), were derived by subcloning the appropriate fragments from pSRNeo-based constructs using the BamHI restriction site. Point mutations within the RING finger domain were introduced by site-directed mutagenesis in the pAlterMAX-HA-Cbl construct using the Altered Sites-II™ mutagenesis system (Promega, Madison, WI) according to the manufacturer’s protocol. The following mutagenic oligonucleotides were used: for mutant C381A/C384A (C3C4), 5′-CTG-GCC-GAG-GCT-GCT-3′; for mutant C401A/C404A (C4C5), 5′-CTG-ACC-GGA-TGT-AAG-AGC-GGA-TGT-GGC-CAT-GAG-GTG-TCC-ACA-3′; for mutant C416A/C419A (C6C7), 5′-CTG-GCC-GAG-GCT-GCT-3′. Notably, the TKB domain of Cbl binds directly to the negative regulatory phosphorylation site in the SH2 kinase linker region of ZAP-70 (Tyr-292) and Syk (Tyr-323) PTKs (11–13). The Tyr → Phe mutations of these sites render ZAP-70 and Syk hyperactive in vivo when expressed in lymphoid cells, whereas the in vitro kinase activity of ZAP-70-Y292F was unchanged (34–36). Similar experiments in a COS-7 cell system demonstrated that co-expression of Cbl induced a loss of the kinase-active pool of Syk, and this effect was accompanied by a decrease in the level of Syk protein (12). These studies have clearly established a critical role for Cbl in the negative regulation of Syk/ZAP-70 PTKs.

Essential Role of RING Finger Domain of Cbl
Importantly, the co-immunoprecipitation analysis revealed that Syk was found to co-immunoprecipitate with the wild type HA-Cbl, and this association was drastically reduced by the G306E mutation. When cell lysates were immunoprecipitated with anti-HA antibody and subjected to anti-Syk immunoblotting, as expected from previous studies (12), the phosphorylation of the indicated pAlterMAX constructs encoding the HA-tagged wild type Cbl (WT), the Cbl-G306E mutant (G306E), or the various truncation mutants of Cbl. Total input DNA was kept constant using the pAlterMAX vector. Cell lysates were prepared after 48 h, and anti-HA immunoprecipitations were carried out from 800 µg of lysate. Whole cell lysates (50 µg in top panel and 10 µg in middle panel) or immunoprecipitated proteins (bottom panel) were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-HA epitope tag 12CA5 (top panel) and antibody (middle and bottom panels) antibodies. Blots were visualized by the ECL method. Arrows on right indicate the positions of Cbl or its mutants and the Syk polypeptide.

**RESULTS**

The N-terminal Region of Cbl, Including the TKB and RING Finger Domains, Is Sufficient for Negative Regulation of Syk—We have previously demonstrated that an intact TKB domain in Cbl and its cognate binding site in Syk are both required for Cbl-dependent negative regulation of Syk (12). However, the isolated TKB domain is dominantly oncogenic and induces an up-regulation of signaling through PTKs such as the PDGFRα (10). These findings suggested that additional C-terminal sequences in Cbl are required for its negative regulatory effect on PTK. To identify the additional domains or regions of Cbl that are necessary for negative regulation of PTKs, we generated a series of C-terminal truncation mutants of Cbl (Fig. 1A) and assessed their effect on Syk.

First, we determined if all of the truncation mutants were expressed as stable proteins and whether these proteins associated with Syk, the latter signifying an intact TKB domain. For this purpose, COS-7 cells were co-transfected with expression plasmids encoding Syk, CD8-ζ (as a scaffold) and various truncation mutants of Cbl. The lysates of transfected cells were immunoblotted directly with anti-HA (for HA-tagged Cbl proteins) and anti-Syk antibodies. Each of the truncation mutants of Cbl was expressed as a polypeptide of expected relative mobility in SDS-PAGE (Fig. 1B, top panel), and all of the Syk-transfected cells expressed the Syk protein (Fig. 1B, middle panel). When cell lysates were immunoprecipitated with anti-HA antibody and subjected to anti-Syk immunoblotting, Syk-HA was found to co-immunoprecipitate with the wild type HA-Cbl, and this association was drastically reduced by the TKB domain-deactivating mutation G306E (Fig. 1B, bottom panel, lanes 3 and 4), as expected from previous studies (12). Importantly, the co-immunoprecipitation analysis revealed that each of the Cbl truncation mutants associated with Syk (Fig. 1B, bottom panel, lanes 5–9).

To assess the functional effects of the truncation mutants of Cbl on Syk, COS-7 cells were transfected with Syk and CD8-ζ, together with graded amounts of Cbl expression plasmids, and whole cell lysates were analyzed by anti-Tyr(P) and anti-Syk immunoblotting. As expected (12), co-expression of increasing amounts of wild type Cbl resulted in a dose-dependent decrease in the tyrosine phosphorylation signal on Syk (Fig. 2, top panel, lanes 2–5). In addition, the phosphorylation of the in vivo substrate CD8-ζ was reduced in a Cbl dose-dependent manner. All of these effects were eliminated by the G306E mutation in Cbl (Fig. 2, 2nd panel, lanes 2–5). Densitometric analysis of Syk protein and phosphotyrosine signals indicated that loss of Syk phosphotyrosine signal was largely due to loss of protein (data not shown).

A reduction of Syk phosphorylation and protein levels as well

![Fig. 1](image-url)
as a reduction in the CD8-ζ phosphorylation were observed upon co-expression with Cbl-655, Cbl-480, or Cbl-436 (Fig. 2, lanes 9–17). Significantly, the Cbl-421 and Cbl-357 mutants failed to have such effects (Fig. 2, lanes 18–23). The deletion in Cbl-421 follows immediately after the last conserved cysteine in the RING finger domain (Cys-419), whereas the Cbl-436 mutant encodes conserved sequences following the RING finger domain (Fig. 1A, also see Fig. 3A). These results demonstrate that, in addition to the TKB domain, residues 358–436 of Cbl are required for negative regulation of Syk and that further C-terminal sequences are dispensable in the present system. Interestingly, the tyrosine phosphorylation of truncation mutants of Cbl was undetectable except for Cbl-421. This finding is consistent with recent results that the predominant Syk phosphorylation sites in Cbl are Tyr-700, Tyr-731, and Tyr-774 (20), which are absent in the truncation mutants. A detectable level of tyrosine phosphorylation on Cbl-421, together with a lack of detectable phosphorylation on Cbl-357 and Cbl-436 mutants, suggests that normally cryptic tyrosine residues may be exposed in this mutant, possibly due to misfolding of the RING finger domain.

The RING Finger Domain of Cbl Is Essential for Negative Regulation of Syk—The analyses presented above indicated that the evolutionarily conserved region of Cbl, including the RING finger domain and the immediate surrounding sequences (Fig. 3A), was involved in the negative regulation of Syk PTK. To assess directly the role of the RING finger domain itself, we introduced substitution mutations in the zinc-coordinating residues of the RING finger domain (Fig. 3B). Previous nuclear magnetic resonance studies of the RING finger domains of the immediate-early protein of equine herpesvirus (42) and the acute promyelocytic leukemia proto-oncoprotein (43) have demonstrated an essential role of the two zinc atoms in stabilizing the RING finger domain structure. Substitution mutations were designed to prevent the coordination of the first (C1C2 and C4C5), the second (C3H and C6C7), or both (C3H/C4C5) zinc atoms (Fig. 3, A and B).

First, we determined if all of the RING finger domain mutants were expressed as stable proteins and whether these proteins associated with Syk. For this purpose, COS-7 cells were co-transfected with expression plasmids encoding Syk, CD8-ζ, and various RING finger domain mutants of Cbl, and lysates of transfected cells were analyzed by anti-HA (for HA-tagged Cbl proteins) and anti-Syk immunoblotting. Each of the RING finger domain mutants of Cbl was expressed as a stable protein with SDS-PAGE mobility similar to that of the wild type Cbl (Fig. 3C, top panel), and all of the Syk-transfected cells expressed the Syk protein (Fig. 3C, middle panel). When cell lysates were immunoprecipitated with anti-HA antibody and subjected to anti-Syk immunoblotting, each of the RING finger domain mutants of Cbl was found to co-immunoprecipitate with Syk, similar to the association of Syk with the wild type Cbl (Fig. 3C, bottom panel, lanes 4–9). Thus, the RING finger domain mutants retained an intact TKB domain.

To assess the effects of the Cbl RING finger domain mutants on Syk, COS-7 cells were transfected with CD8-ζ and Syk together with graded amounts of plasmids encoding the RING finger domain mutants, and lysates of transfected cells were analyzed by anti-Tyr(P) and anti-Syk immunoblotting. As expected, the expression of wild type Cbl, but not Cbl-G306E, led to a dose-dependent decrease in the phosphorylation and protein level of Syk. Significantly, none of the RING finger mutants tested was capable of decreasing the phosphorylation and protein level of Syk. In fact, a Cbl dose-dependent increase in the Syk phosphorylation and protein level was observed in lysates of cells transfected with RING finger mutants, even though the levels of mutant Cbl proteins were similar to or lower than those of the wild type Cbl (Fig. 4A, top and bottom panel, lanes 9–23). The level of tyrosine phosphorylation on transfected RING finger domain mutant Cbl proteins was also higher compared with that on wild type Cbl or on the Cbl-G306E mutant (Fig. 4A, top and middle panel, lanes 9–23).

To characterize further the effect of the RING finger domain mutants on Syk, an in vitro kinase assay was performed on anti-Syk immunoprecipitates. The kinase activity was quantified by assessing [γ-32P]ATP incorporation into the Raytide peptide substrate as well as by autoradiographic assessment of the autophosphorylation of Syk (Fig. 4, B–D).

As anticipated, the co-expression of Syk with wild type Cbl led to a Cbl dose-dependent decrease in the level of Raytide phosphorylation as well as Syk autophosphorylation, whereas the Cbl-G306E mutant did not have these effects (Fig. 4, B–D). In contrast, a dose-dependent increase in the Syk kinase activity was observed in immunoprecipitates derived from COS-7
cells transfected with the RING finger domain mutants of Cbl. This enhancement was revealed in both Raytide phosphorylation (1.6–2.2-fold increase compared with 84% maximal reduction with wild type Cbl) and autophosphorylation assays (1.7–2.2-fold increase compared with 60% maximal reduction with wild type Cbl) (Fig. 4, B–D). Together with the results of Fig. 4A, the in vitro kinase assays directly demonstrated that the RING finger domain mutants of Cbl had lost the ability to reduce the kinase-active pool of Syk in transfected COS-7 cells.

The RING Finger Domain Is Required for Cbl to Inhibit the Syk-dependent SRE Reporter Transactivation—By having defined the Cbl RING finger domain as a critical element for Cbl-dependent negative regulation of Syk activity, we wished to examine the consequences of this regulation on a distal readout of PTK activation. For this purpose, we examined the effects of wild type Cbl and various Cbl mutants on the transactivation of the serum response element (SRE) derived from COS-7 cells. COS-7 cells transfected as in Fig. 1B. Each plate received 0.5 μg of pSRneo-CDS-ζ, 0.05 μg of Syk, and 1 μg of the indicated pAlterMAX construct encoding the HA-tagged wild type Cbl (WT), Cbl-G306E mutant (G306E), or the indicated RING finger domain mutants of Cbl (see B for nomenclature). Total input DNA was kept constant using the pAlterMAX vector. Cell lysates were prepared 48 h post-transfection, and anti-HA immunoprecipitations were carried out from 800 μg of lysate. Whole cell lysates (50 μg in top panel and 10 μg in middle panel) or immunoprecipitated proteins (bottom panel) were subjected to immunoblotting with anti-HA (top panel) and anti-Syk (middle and bottom panels) antibodies. Blots were visualized using the ECL method. Arrows on right indicate the positions of Cbl or its mutants and the Syk polyepitide.

of 1. Expression of Syk resulted in a 3–3.6-fold induction of the SRE luciferase activity compared with that in mock-transfected cells (Fig. 5). Significantly, the co-expression of the wild type Cbl reduced the SRE luciferase activity by 45%, and this effect was abrogated by the G306E mutation, consistent with the critical role of the TKB domain (12). A significant reduction of the SRE luciferase activity was observed upon co-expression of Cbl-436, consistent with its effects on the levels of Syk phosphorylation and protein levels (Fig. 2). In contrast, Cbl proteins with further truncation (Cbl-421 and Cbl-357) or specific RING finger domain mutations (C3HC4C5 and C1C2) failed to exert any inhibitory effects on the SRE luciferase activity; in fact certain mutants (e.g. Cbl-421 and Cbl-357) led to an enhancement of the SRE luciferase reporter activity. These results in the 293T transfection system further establish that, in addition to the TKB domain, an intact RING finger domain is required for Cbl-dependent negative regulation of the Syk PTK.

The RING Finger Domain of Cbl Is Required for Regulation of the Protein Level of Syk in Human B-lymphoma Cell Line Ramos upon Anti-IgM Stimulation—To confirm the effects of Cbl on Syk PTK levels observed in COS-7 and 293T reconstitution systems in a more physiological setting, we utilized the Ramos B-lymphoblastic cell line. These cells have been widely used to assess B-lymphocyte receptor signaling in which Syk plays as essential role (44). To allow transient expression, we first generated an SV40 T antigen-expressing derivative of this line designated Ramos-T. These cells were transfected with
either pAlterMAX, pAlterMAX-HA-Cbl, or pAlterMAX-HA-Cbl-C3HC4C5C6, and lysates of transfected cells were prepared with or without anti-IgM stimulation for various time points. These lysates were subjected to immunoblotting with anti-Tyr(P) (top panel), anti-Syk (middle panel), or anti-HA (bottom panel) antibodies and visualized using the ECL method. The Cbl construct designations are as shown in Fig. 3B. Arrows on right indicate the positions of Cbl or its mutants and the Syk polypeptide. ●, Cbl WT; ■, Cbl G306E; ▲, C1C2; □, C3H; △, C4C5; ●, C6C7; [irco], C3HC4C5. B–D, anti-Syk immunoprecipitations were carried out from 800 μg of the same lysates as used in A. Immunoprecipitated Syk proteins were subjected to an in vitro kinase reaction in the presence of the substrate Raytide. After 30 min incubation at 30 °C, the supernatant was used to measure the incorporation of 32P-labeled ATP into Raytide using Cerenkov counting. The values of Raytide phosphorylation were plotted as a ratio to that in the sample without Cbl (value of 1) and are shown in B. The autophosphorylated Syk protein bound to protein A-Sepharose was resolved by SDS-PAGE and detected by autoradiography (C). Densitometric quantification analysis of Syk bands shown in C was carried out and is plotted as relative level of Syk auto-phosphorylation in D. The values without Cbl co-expression were taken as 1.

**DISCUSSION**

The Cbl proto-oncogene product has emerged as a potential negative regulator of both receptor and non-receptor tyrosine kinases, including the Syk/ZAP-70 PTKs that play essential roles in lymphocyte antigen receptor signal transduction and provide eminent models of non-receptor PTK-mediated cellular activation (1, 2, 6, 45, 46). Here, we demonstrate that, in addition to the previously reported role of the TKB domain, the RING finger domain of Cbl is essential for its negative regulatory effect on the Syk PTK. Furthermore, the evolutionarily conserved N-terminal half of Cbl, incorporating the TKB and RING finger domains, is sufficient for Cbl-dependent negative regulation of Syk.
cell systems are complementary. However, whereas the RING finger domain mutants increased both phosphorylation and protein levels of Syk in COS-7 cells, we did not see an enhancement of SRE luciferase activity in 293T system. Nonetheless, the inhibitory effect of Cbl on Syk was abrogated by the RING finger mutations in both systems.

Notably, the RING finger domain and the immediate flanking sequences represent the most conserved region among Cbl family proteins, more so than even the TKB domain that was previously demonstrated to be essential for Cbl function. The RING finger domain, originally identified in RING (Really Interesting Novel Gene)-1, a polypeptide of unknown function, has now been identified in over 80 proteins (47, 48). Recent studies have implicated this domain as an important element in the function of various proteins. For example, mutations designed to inactivate the zinc binding ability of the RING finger domain severely reduced the transforming activity of the leukemia-derived chimeric oncogene rfp/ret in which the RING finger from rfp gene is fused to the tyrosine kinase domain of Ret receptor (49). Similarly, point mutations or deletion of the N-terminal RING finger domain of Siah-1 protein abrogated its ability to promote proteolysis of the DCC (deleted in colon cancer) tumor suppressor protein (50). Our analyses provide a clear example of a critical role played by the RING finger domain in the biological function of Cbl as a negative regulator of PTKs. Recent findings indicate that the RING finger domains may mediate binding of ubiquitin-conjugating enzymes (E2s) (51), suggesting that the Cbl RING finger domain might recruit E2 components of the ubiquitination machinery to target tyrosine kinases. Interestingly, Cbl fusion proteins could enhance the ubiquitination of EGF receptor in vitro in whole rabbit reticulocyte lysate, and the RING finger was required for this effect (52).

Fig. 5. The RING finger domain is essential for the inhibitory effect of Cbl on Syk tyrosine kinase-dependent transactivation of the SRE luciferase reporter. 1.0 × 10⁵ 293T cells were plated per well of a 6-well dish. After overnight culture, the cells were transfected using the calcium-phosphate transfection method. Each well received 1 μg of the SRE luciferase reporter together with either the vector alone (Mock) or 0.01 μg of the pAlterMAX-Syk plasmid with or without 0.6 μg of the pAlterMAX constructs encoding the wild type HA-Cbl (Cbl-WT) or its mutants. Cell lysates were prepared 48 h post-transfection. The luciferase activity was determined on equal aliquots of cell lysates, and results are expressed as a fold increase relative to the mock transfectant (no Cbl or Syk). Data points represent the mean ± 1 S.D. of 5 replicate transfections.

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How might the RING finger domain contribute to Cbl function? At present, there is no general paradigm for the role of RING finger domains. Given their occurrence in a large array of proteins with diverse functions, domain structures, and subcellular localization, the RING finger domain is likely to be an independent structural module. By analogy to other modular domains found in signaling proteins, it is reasonable to suggest that the RING finger domains may mediate protein-protein, protein-lipid, or other molecular interactions. However, a shared ligand for the RING finger domains has not been identified to date. Interestingly, a related two zinc atom-coordinat-
ing domain in the early endosome antigen protein, the FYVE domain, binds to phosphatidylinositol-3-OH kinase product PIP3 (53), and the RING finger domain of promyelocytic leukemia proto-oncoprotein associates with the E2-like ubiquitin-conjugating enzyme, UBC9 (54). Further studies are clearly warranted to determine if the RING finger domain of Cbl and other proteins provide an interface for similar interaction with other macromolecules.

Given recent findings on the effects of Cbl on receptor PTKs, a potential role of the RING finger domain in linking Cbl to ubiquitination and/or proteasomal pathway appears plausible. Cbl overexpression leads to enhanced ubiquitination, down-regulation from the cell surface, and degradation of the PDGFR and EGFR (28, 30, 31). These effects require the TKB domain, and experiments in the EGFR system indicate that the TKB domain alone is not sufficient, suggesting the role of additional Cbl sequences. Given the evolutionary conservation of the RING finger domain, it is likely to play a role in Cbl-dependent ubiquitination and down-regulation of receptor PTKs. Whether or not the reduction in Syk protein levels observed in the COS-7 cell system is a reflection of such a mechanism remains to be determined.

Previous studies have revealed that the naturally occurring 70Z/3 Cbl mutant, with deletion of aa 366–382 (in the human equivalent), is oncogenic and induces the up-regulation of the sequences present in the C-terminal half of the protein. An equivalent of the minimal Syk inhibitory region of Cbl (from aa 366–382) is oncogenic and induces the up-regulation of the negative regulatory function of Cbl on PTKs may not require the first cysteine residue that is expected to participate in zinc coordination.

Surprisingly, truncation analyses in the COS-7 cell reconstitution system revealed that the C-terminal sequences (residues 437–906) were dispensable for the negative regulation of Syk by Cbl. These effects were confirmed in 293T cells using the SRE luciferase reporter assay. These findings suggest that the negative regulatory function of Cbl on PTKs may not require the sequences present in the C-terminal half of the protein.

These findings are in line with the negative regulatory effects of SLP-76 and Dbl on the EGFR. Notably, Dbl is structurally an equivalent of the minimal Syk inhibitory region of Cbl identified here. These findings lead us to suggest that the reduction of the kinase-active pool of PTKs may be a core function of Cbl proteins that is mediated through the N-terminal region, which includes the TKB and RING finger domains. However, the interactions mediated via the C-terminal sequence may modify this core function. For example, mutation of the tyrosine phosphorylation sites accentuated the phenotype of 70Z/3 Cbl mutant to transactivate the NF-AT luciferase reporter in transfected Jurkat cells, suggesting a role for these sites in the negative regulatory effect of Cbl. However, further studies are needed to understand the better the mechanism by which the interaction of the C-terminal part of Cbl might influence Cbl function and the manner in which these interactions might affect the function of the more conserved N-terminal domains.

In conclusion, our studies provide evidence that the RING finger domain of Cbl is essential for the negative regulation of Syk PTK and that the RING finger and TKB domains together are sufficient for this function. These findings, together with the evolutionary conservation of the TKB and the RING finger domains, define the elements of Cbl family proteins that are crucial for their core function as negative regulators of PTKs. Further studies designed to assess the biochemical function(s) of the RING finger domain are likely to yield clues about the mechanism of negative regulation of PTKs by the Cbl family proteins.
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