Cbl-mediated Negative Regulation of the Syk Tyrosine Kinase

A CRITICAL ROLE FOR Cbl PHOSPHOTYROSINE-BINDING DOMAIN BINDING TO Syk PHOSPHOTYROSINE 323*

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The proto-oncogene product Cbl has emerged as a potential negative regulator of the Syk tyrosine kinase; however, the nature of physical interactions between Cbl and Syk that are critical for this negative regulation remains unclear. Here we show that the phosphotyrosine-binding (PTB) domain within the N-terminal transforming region of Cbl (Cbl-N) binds to phosphorylated Tyr323 in the linker region between the Src homology 2 and kinase domains of Syk, confirming recent results by another laboratory using the yeast two-hybrid approach (Deckert, M., Elly, C., Altman, A., and Liu, Y. C. (1998) J. Biol. Chem. 273, 8867–8874). A PTB domain-inactivating point mutation (G306E), corresponding to a loss-of-function mutation in the Caenorhabditis elegans Cbl homologue SLI-1, severely compromised Cbl-N/Syk binding in vitro and Cbl/Syk association in transfected COS-7 cells. Using heterologous expression in COS-7 cells, we investigated the role of Cbl PTB domain binding to Syk Tyr323 in the negative regulation of Syk. Co-expression of Cbl with Syk in COS-7 cells led to a dose-dependent decrease in the autophosphorylated pool of Syk and in phosphorylation of an in vivo substrate, CD8-ζ. Unexpectedly, these effects were largely due to the loss of Syk protein. Both the decrease in Syk and CD8-ζ phosphorylation and reduction in Syk protein levels were blocked by either G306E mutation in Cbl or by Y323F mutation in Syk. These results demonstrate a critical role for the Cbl PTB domain in the recruitment of Cbl to Syk and in Cbl-mediated negative regulation of Syk.

Studies over the last 4 years have now firmly established

that Cbl, the product of the c-cbl proto-oncogene, is a component of early tyrosine kinase signaling cascades in a variety of cell types (reviewed in Refs. 1 and 2). For example, Cbl is an early and prominent target of tyrosine phosphorylation in response to activation of a number of cell surface receptors coupled to tyrosine kinase activation, including the lymphocyte antigen receptors, cytokine receptors, and growth factor receptors (1, 2). Cbl’s extensive proline-rich region (amino acids 481–690) mediates interactions with the SH3 domains of Src family kinases and adapter proteins Grb2 and Nck to promote the formation of signaling protein complexes that are present in cells prior to receptor activation. Receptor activation-dependent phosphorylation of Cbl on tyrosine residues creates docking sites for several SH2 domain-containing proteins (3–5), leading to activation-induced association of Cbl with the p85 subunit of phosphatidylinositol 3-kinase, the Crk adapter proteins, and VAV (1, 2). Finally, we showed that the transforming N-terminal region of Cbl (Cbl-N, equivalent to Cbl sequences present in the v-cbl oncogene) harbors a phosphotyrosine-binding domain that selectively and directly interacts with the T cell tyrosine kinase Zap-70 (6, 7). Subsequently, this domain was also shown to mediate binding to receptor tyrosine kinases EGFR and PDGFRα (1, 2, 8–10). While these associations have promoted the notion that Cbl functions as a complex adapter protein that couples tyrosine kinases to downstream signaling pathways, a number of recent biochemical and genetic studies have identified Cbl as a potential negative regulator of Syk/ZAP-70, as well as other PTKs (1, 2).

The C. elegans homologue of Cbl, SLI-1, was identified through a genetic screen as a negative regulator of the LET-23 receptor tyrosine kinase, a homologue of the mammalian EGFR, which functions in the vulval development (11). Notably, the loss of function mutations in SLI-1 were mapped to the evolutionarily conserved N-terminal region, corresponding to the PTB domain. One of these was a point mutation (G315E); the analogous mutation in mammalian Cbl-N abrogated its PTB domain activity (6). Recently, a Drosophila Cbl homologue (D-Cbl) was identified and also shown to function as a negative regulator of the Drosophila EGFR-mediated R7 photoreceptor development (12, 13). Dominant oncogenic forms of mammalian Cbl introduced into NIH-3T3 cells induced an up-regulation of signaling downstream of the endogenous PDGFRα tyrosine

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¶¶¶ The abbreviations used are: SH2 and -3, Src homology 2 and 3, respectively; Tyr(T) or pY, phosphotyrosine; CD, cluster of differentiation; mAb, monoclonal antibody; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; EGFR, epidermal growth factor receptor; PDGFRα, platelet-derived growth factor receptor α; HA, hemagglutinin; PTB, phosphotyrosine-binding; mAb, monoclonal antibody.
The ability of Cbl to function as a tyrosine kinase regulator has heightened the need to determine the nature of its physical and functional interactions with this class of enzymes. Studies in our laboratory and others have focused on the interactions of Cbl with the Syk/ZAP-70 family of tyrosine kinases, critical elements in cellular activation through antigen receptors. Cbl was shown to associate with Syk and ZAP-70 in lymphocytes activated, respectively, through the B cell receptor or T cell receptor (17, 18). Notably, Cbl/Syk association was also observed in a myeloid leukemic cell line and in RBL-2H3 mast cells expressing Syk and Cbl; however, significant Cbl/Syk association was observed prior to FcεR or FcεR1 ligation (19, 20). Cbl was shown to serve as a substrate for Syk or ZAP-70 when co-expressed with these proteins in COS cells (21) or when Syk was expressed as the cytoplasmic tail of an IgFv-Syk construct. These results suggest that Cbl is a protein that can bind to Syk and ZAP-70 as early as in the cytoplasmic domain of Syk, prior to its transmembrane domain.

Activation of Ramos B Cells—Cells were washed and resuspended in RPMI 1640 medium with 20% FCS, pH 7.2, at 10^7/ml and preincubated for 30 min at 37 °C for 2 min in the presence of an anti-human IgM (clone OX-2) antibody at 10 μg/ml. Cbl or Syk mutants, and GST fusion proteins were added to the cells in a concentration of 20 μg/ml. Cells were incubated for the indicated time points and directly lysed by adding three volumes of cold lysis buffer consisting of 0.5% Triton X-100 (Fluka); 50 mM Tris (pH 7.5 at room temperature); 150 mM sodium chloride; 1 mM phenylmethylsulfonyl fluoride; 1 μg/ml each of leupeptin, pepstatin A, antipain, and chymostatin; 1 mM sodium orthovanadate; and 10 mM sodium fluoride (Sigma).

Transient Expression in COS Cells—COS-7 cells were transfected for 6 h using Lipofectamine™ (Life Technologies, Inc.) in OPTI-MEM medium following the manufacturer's protocol. For Fig. 4, DNA concentrations were 5 μg of Cbl or Cbl-G306E, 2 μg of Syk or Syk mutants, and 1 μg of CD8-β plasmids for 1.6 x 10^7 cells/10-cm dish. For Fig. 5, DNA concentrations were 1.2–2.0 μg of Cbl or Cbl-G306E as described (6). The following mutagenic oligonucleotides were used for site-directed mutagenesis: G306E, 5'-GGT-AGT-AAC-ATA-CTC-ATC-AGC-CCA-3; Y323F, 5'-AGT-AAC-ATA-CTC-AAT-AGC-CCA-3. The sequence fidelity of all constructs was verified by automated DNA sequencing.

Binding Reactions, Immunoprecipitations, Gel Electrophoresis, and Immunoblotting—Binding reactions of 20 μg GST fusion proteins and extracts of 2.5 x 10^7 Ramos cells and immunoprecipitations from 1 x 10^7 Ramos or 2 mg of COS-7 cell lysates were performed as described (6, 18). Bound proteins and total cell lysates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Haverhill, MA), and immunoblotted with the indicated antibodies. Blots were visualized using enhanced chemiluminescence (ECL; reagents from NEN Life Science Products). Blots were stripped and reprobed, as described (6). Photographs of blots were generated by direct scanning of films using a Hewlett Packard Scanjet 4c scanner and Corel Draw TM version 6 software. Densitometry was carried out on
Fig. 1. The PTB domain-containing N-terminal region of Cbl (Cbl-N) selectively and directly binds to Syk, and binding is abrogated by the G306E mutation. A, Ramos B cells were either left unstimulated (−) or stimulated with an anti-IgM antibody for 10 min (+) prior to lysis. Binding reactions (Binding Rxn) and immunoprecipitations (I.P.) from lysates of $2.5 \times 10^7$ cells were resolved by SDS-PAGE, and the membrane was immunoblotted with an anti-Tyr(P) antibody (top part) followed by an anti-Syk (bottom part) antibody. The blots were developed using a protein A-horseradish peroxidase conjugate and ECL. NRS, normal rabbit serum; aSyk, anti-Syk antibody; IgH, heavy chain of immunoglobulin. B, binding reactions with GST or GST-Cbl-N fusion proteins were carried out as in A, except that Ramos B cells were stimulated with anti-IgM antibody for the indicated times (−, no stimulation; ′, minutes). C, anti-Syk immunoprecipitations from lysates of $5 \times 10^7$ Ramos B cells stimulated for 10 min with anti-IgM antibody were resolved by SDS-PAGE, transferred to PVDF membranes, and incubated with a 2.5 μg/ml concentration of the indicated soluble GST fusion proteins, followed by serial incubation with an anti-GST antibody (aGST), protein A-horseradish peroxidase, and ECL detection (lane 1). Membranes were then stripped and serially reprobed with anti-Tyr(P) (lane 2) and anti-Syk (lane 3) antibodies.

RESULTS

Cbl Binding to Syk Is Mediated by the Cbl PTB Domain—Given the activation dependence of the association between Syk and Cbl (Ref. 15 and data not shown) and the finding that similar activation-dependent association between ZAP-70 and Cbl is mediated by the PTB domain in the Cbl-N region (6, 7), we first examined the ability of Cbl-N to bind to Syk in vitro. For this purpose, lysates were prepared from Ramos human B cells that were either left unstimulated or were stimulated with an anti-IgM antibody for 10 min. These lysates were incubated with glutathione- Sepharose beads coated with GST, GST-Cbl-N, or GST-Cbl-N-G306E fusion protein. Bound polypeptides were resolved by SDS-PAGE along with control or anti-Syk immunoprecipitates prepared from the same lysates, transferred to a PVDF membrane, and immunoblotted with anti-Tyr(P) and anti-Syk antibodies (Fig. 1A).

A 72-kDa phosphotyrosyl polypeptide was the most prominent band observed in GST-Cbl-N binding reactions (Fig. 1A, lanes 3 and 4, upper part), and this band comigrated with directly immunoprecipitated tyrosine-phosphorylated Syk (lanes 9 and 10, upper part). Reprobing of the blot identified the 72-kDa polypeptide as Syk (lanes 3 and 4, lower part). These analyses show that Syk specifically binds to GST-Cbl-N, as no signal was observed in GST binding reactions (lanes 1 and 2, upper and lower parts). Binding of Cbl-N to Syk in lysates of unstimulated cells was low, but it increased substantially upon anti-IgM stimulation of the cells (compare lanes 3 and 4). Importantly, a PTB domain-inactivating point mutation in Cbl-N (Cbl-N-G306E) completely abrogated binding to Syk (lanes 5 and 6, upper and lower parts). Notably, while the amount of Syk protein bound to Cbl-N was modest, the phosphotyrosine signal was substantial when compared with directly immunoprecipitated Syk (compare lanes 4 and 10, upper part). In addition, the amount of Syk bound to Cbl-N in lysates...
varied with the time of stimulation of cells prior to lysis (Fig. 1B). This binding closely paralleled the level of Syk tyrosine phosphorylation observed in lysates and anti-Syk immunoprecipitations (data not shown).

These results establish that Cbl-N interacts with Syk in a lysis-binding assay. However, it was possible that this interaction was indirectly mediated by an adapter molecule. To determine if the Cbl PTB domain directly bound to Syk, a far Western blot was performed. Anti-Syk immunoprecipitations were carried out using lysates of anti-IgM-activated Ramos cells; proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Replicate blots were incubated with the indicated GST fusion proteins. Bound fusion proteins were detected with an anti-GST antibody (Fig. 1C, lane 1). The membranes were then stripped and serially probed with anti-Tyr(P) (lane 2) and anti-Syk (lane 3) antibodies. As seen in Fig. 1C, GST-Cbl-N directly bound to Syk; in contrast, no binding was observed with GST or GST-Cbl-N-G306E, although each of the probed membranes carried similar amounts of tyrosine-phosphorylated Syk. These data demonstrate that the Cbl PTB domain can directly bind to the Syk tyrosine kinase.

To rule out the possibility that the Cbl-N/Syk binding may be specific to the Ramos B cell line, binding reactions were also carried out using lysates of the WEHI-231 murine B cell line and the Jurkat-JMC T cell line, both of which express the Syk tyrosine kinase. GST-Cbl-N but not its G306E point mutant bound prominently to both Syk and ZAP-70 in lysates of anti-CD3 stimulated Jurkat-JMC cells and to Syk in lysates of anti-IgM-stimulated WEHI-231 cells (data not shown). While the above analyses clearly demonstrated the capability of Cbl-N to bind to Syk, it remained possible that other regions within Cbl also contribute to the Cbl/Syk interaction. To address this possibility, we compared the Syk binding capabilities of Cbl-N (amino acids 1–357) and Cbl-C (amino acids 358–906), the latter containing the RING finger domain, the proline-rich region, and the leucine zipper of Cbl. Compared with the prominent binding of GST-Cbl-N to Syk in lysates of activated Ramos B cells (Fig. 2A, lanes 5 and 6, upper part), GST-Cbl-C failed to bind to Syk in lysates of both unstimulated and anti-IgM-stimulated Ramos cells (lanes 3 and 4, upper part). Similar results were observed using lysates from anti-IgM-stimulated WEHI-231 B cells and anti-CD3-stimulated Jurkat T cells (data not shown). Importantly, the GST-Cbl-C protein was competent to bind other proteins, as demonstrated by its activation-independent binding to Grb2 (lanes 3 and 4, lower part), an association mediated by the Grb2 SH3 domain binding to the Cbl proline-rich region (1, 2). Neither GST-Cbl-N nor GST alone bound to Grb2 under any condition (Fig. 2A, lanes 1 and 2 and lanes 5 and 6, lower part).

Given the prominent activation-induced tyrosine phosphorylation of Cbl upon B cell receptor ligation (Ref. 18 and data not shown), it was formally possible that the SH2 domains of Syk mediate binding to Cbl. To directly test this possibility, we examined the ability of GST fusion proteins containing various combinations of the Syk SH2 domains and the SH2-kinase interdomain linker to bind to Cbl in lysates of unstimulated (−) or anti-IgM-stimulated (+) Ramos B cells. GST-Crk-SH2, used as a known Cbl-binding control (28), prominently bound to Cbl in lysates of stimulated but not unstimulated Ramos cells (Fig. 2B, lanes 3 and 4). In contrast, none of the Syk SH2 domain-containing GST fusion proteins, with or without the SH2-kinase interdomain linker region, were able to bind to Cbl (Fig. 2B, lanes 5–12). Taken together, the results presented above demonstrate that Cbl-N binds to Syk and thus may mediate the observed anti-IgM-stimulation-induced association between Cbl and Syk. The ability of GST-Cbl-N but not its G306E mutant to bind to Syk indicated that the Syk/Cbl interaction was mediated by the Cbl PTB domain. In further support of this conclusion, Cbl-N binding to Syk was abrogated by millimolar concentrations of free phosphoryrosyne and by micromolar concentrations of a phosphothreonyl peptide corresponding to the Cbl PTB domain binding site on ZAP-70 (Tyr(P)292; IC_{50}, 5 μM; data not shown).

Identification of a Cbl PTB Domain Binding Site on Syk—The ability of the Cbl PTB domain to bind to Syk suggested that Syk must possess a Cbl PTB domain-binding motif that is induced by autophosphorylation. Comparison of the primary amino acid sequence of human Syk with the determined Cbl PTB domain binding motif, D(N/D)pY, revealed that six tyrosine residues within Syk were in the appropriate context to serve as potential binding sites: FYNPP^{23} (Tyr^{23}), DNPY^{31} (Tyr^{31}), IKNY^{109} (Tyr^{109}), DENYY^{208} (Tyr^{208}), CINYY^{547} (Tyr^{547}), and RNY^{230} (Tyr^{230}) (8, 27). Three of these tyrosine residues (Tyr^{23}, Tyr^{52}, and Tyr^{208}) corresponded to defined in vitro autophosphorylation sites on murine Syk (32), and Tyr^{230} corresponds to ZAP-70 Tyr^{292}, the defined Cbl PTB domain binding site on ZAP-70 (7). In order to determine if any of the known autophosphorylation sites on Syk can serve as a Cbl PTB domain-binding site, 17-mer synthetic phosphopeptides incorporating 8 amino acids on either side of the phosphothreonyl residue were generated and examined for competition of GST-Cbl-N binding to Syk in lysis-binding reactions. As expected, the phosphorylated ZAP-70 Tyr^{292} phosphopeptide (10 μM) efficiently inhibited Syk binding to GST-Cbl-N (Fig. 3, lane 5), whereas the corresponding nonphosphorylated peptide (Tyr^{292}) had no effect even at 30 μM concentration (lane 4). Syk-Tyr^{323} (lanes 6–8) as well as Syk-Tyr^{230} (lanes 9–11) phosphopeptides inhibited Syk binding to GST-Cbl-N with similar IC_{50} values (5.3 μM versus 6 μM, respectively, as determined by densitometry; data not shown). These in vitro binding data suggested that one or both of the corresponding phosphorylation sites on Syk could serve as in vivo binding sites(s) for the Cbl PTB domain.
domain. We were unable to test a Tyr\textsuperscript{630} phosphopeptide in competition experiments due to its extreme insolubility.

To assess the role of the Tyr\textsuperscript{323}, Tyr\textsuperscript{526}, and Tyr\textsuperscript{630} autophosphorylation sites in Cbl/Syk association in \textit{vivo}, Syk mutants with tyrosine to phenylalanine substitutions were generated. Wild type or mutant Syk constructs were co-transfected into COS-7 cells along with HA-tagged wild type Cbl and a CD8-\zeta chimera, using conditions comparable with those previously shown to lead to expression of active Syk protein in COS cells (33). Consistent with the previous study (33), activation of Syk in COS cells required the co-expression of the immunoreceptor tyrosine activation motif-containing CD8-\zeta chimera but did not require a co-expressed Src family kinase (data not shown). Lysates of cells transfected in this manner were subjected to anti-HA immunoprecipitation followed by anti-Syk or anti-Grb2 immunoblotting to assess the association of introduced Cbl with the co-transfected Syk or with endogenous Grb2 (the latter association is via Cbl’s proline-rich regions).

Anti-HA and anti-Syk immunoblotting of whole cell lysates demonstrated that comparable levels of all Syk constructs and Cbl were expressed under the experimental conditions (Fig. 4A, \textit{third} and \textit{fourth} parts). As anticipated, Cbl showed prominent association with Grb2; this interaction was not significantly affected by co-expression of Syk or its mutants (Fig. 4A, \textit{second part}). Notably, wild type Syk co-immunoprecipitated with wild type Cbl; this association was markedly reduced by the Syk-Y323F mutation (comparing lane 2 with lane 3, \textit{first part}), strongly suggesting that phosphorylated Tyr\textsuperscript{323} represents an important \textit{in vivo} binding site for the Cbl PTB domain. Since binding was not completely abolished by the Y323F mutation, an additional secondary Cbl-binding site may also exist on Syk. Alternatively, the Cbl PTB domain may still bind with a certain affinity to the mutated Tyr\textsuperscript{323} site. Notably, Cbl binding to Syk-Y323F was further reduced by the G306E mutation (data not shown). Additional mutations will be necessary to precisely distinguish between these alternative explanations.

Interestingly, the Syk-Y525F/Y526F mutation also resulted in reduced Cbl/Syk association (Fig. 4A, lane 4, \textit{upper part}). While this result suggests that Tyr\textsuperscript{526} may serve as a secondary binding site for the Cbl PTB domain, the Y525F/Y526F mutation severely reduced the tyrosine phosphorylation of Syk in COS-7 cells (Fig. 4A, \textit{fifth part}, lane 4) and is known to reduce the catalytic activity of Syk (34). Therefore, reduced Cbl PTB domain binding to Syk-Y525F/Y526F may reflect the role of Tyr\textsuperscript{526} and Tyr\textsuperscript{525} in Cbl PTB domain binding, an indirect effect due to reduced phosphorylation of Tyr\textsuperscript{525}, or both. The Syk-Y629F/Y630F/Y681P mutation did not reduce the Cbl/Syk association and in fact led to a slight increase in association (lane 6, \textit{first part}). While the mechanism for this enhancement is not clear, this result strongly argues against the Cbl PTB domain binding to the Tyr\textsuperscript{630} site.

In an additional experiment, we compared the association of Syk with wild type Cbl versus a PTB domain mutant, Cbl-G306E. This analysis showed that the association of Syk with Cbl was markedly decreased by the G306E mutation of Cbl (Fig. 4B, compare lanes 2 and 3, \textit{upper part}). These results showed that an intact Cbl PTB domain is required for the \textit{in vivo} association between Cbl and Syk in the COS cell system. Taken together, our results clearly indicate that Syk Tyr\textsuperscript{323} represents a major Cbl PTB domain-binding site on Syk and contributes to \textit{in vivo} Cbl/Syk association. While this manuscript was in preparation, Deckert \textit{et al.} (23) reported a similar conclusion based on a yeast two-hybrid interaction between Cbl and Syk.

### Fig. 3. Competition of Cbl-N binding to Syk by Syk-derived phosphopeptides

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### Fig. 4. Identification of Tyr\textsuperscript{323} as the major Cbl PTB domain binding site on Syk

**A**

- **Vector**
- **Cbl**
- **αHA I.P. αSyk Blot**
- **αHA I.P. αGrb2 Blot**
- **Lysate αSyk Blot**
- **Lysate αTyr(P) Blot**

**B**

- **Cbl**
- **αHA I.P. αHA Blot**
- **Lysate αSyk Blot**
- **αHA Blot**

This figure shows the binding of Syk to Cbl and the role of Tyr\textsuperscript{323} in this interaction. The binding of Syk to Cbl was assessed by immunoprecipitation and blotting with anti-Syk antibody, and the role of Tyr\textsuperscript{323} in this interaction was confirmed by the reduced binding of Syk to Cbl-G306E.
Cbl PTB Domain Binding to Syk Tyr323 is required for Cbl-mediated Negative Regulation of Syk—Although the Syk Y323 is analogous to the Tyr292 negative regulatory autophosphorylation site in ZAP-70 (31), the role of Syk Tyr323 has not been directly assessed. In view of the results presented above, we reasoned that Cbl PTB domain binding to Syk Tyr323 may be involved in Cbl-mediated negative regulation of the Syk tyrosine kinase.

To investigate this possibility, we co-expressed wild type Syk or Syk-Y323F with increasing amounts of HA-Cbl or HA-Cbl-G306E and a constant amount of CD8-ζ (which provides an in vivo substrate for Syk in addition to its function as a scaffold for the activation of Syk) in COS-7 cells. However, the amount of Syk expression vector DNA used for transfection was decreased (0.05 μg/6-cm dish) compared with that used in Fig. 4, A and B (4 μg/10-cm dish). This was based on initial experiments to define conditions for optimal inhibition of Syk autophosphorylation when it was co-expressed with Cbl (data not shown). 48 h after transfection, total cell lysates were immunoblotted with anti-Tyr(P), anti-HA, anti-Syk, and anti-ζ mAbs (Fig. 5A, first panel, compare lanes 1–5 with lane 16). Under these conditions, comparable levels of wild type Cbl and Cbl-G306E were expressed, although at equivalent input DNA concentrations the Cbl-G306E protein levels were higher compared with Cbl levels (Fig. 5A, second panel).

Co-expression of Syk with increasing amounts of HA-Cbl led to an increasing reduction in the level of tyrosine-phosphorylated Syk and tyrosine-phosphorylated CD8-ζ detectable in cell lysates (Fig. 5A, first part, compare lanes 6–10 with lane 16). This effect was not seen when the Y323F mutant of Syk was coexpressed with wild type Cbl (Fig. 5A, first panel, compare lanes 1–5 with lane 16). Furthermore, expression of increasing amounts of the PTB-domain mutant Cbl-G306E failed to induce a decrease in the signals of tyrosine-phosphorylated Syk or tyrosine-phosphorylated CD8-ζ (first panel, compare lanes 11–15 with lane 16). Thus, both a functional Cbl PTB domain and an intact Syk Tyr323 motif were required for Cbl-induced reduction of the kinase active pool of Syk in COS-7 cell system.

To determine if the decrease in phosphorytosine signals on Syk was due to a decrease in the level of phosphorylation or a decrease in the level of protein, anti-Syk immunoblotting of cell lysates (Fig. 5A, third panel, compare lanes 6–15 with lane 16) and an intact Syk Tyr323 motif were required for Cbl-induced reduction of the kinase active pool of Syk in the COS cell system. Furthermore, the levels of Cbl-induced reduction in phosphorytosine signals on Syk was accompanied by a similar reduction in the level of Syk protein in the lysates (Fig. 5A, third part, compare lanes 6–15 with lane 16). Importantly, the level of Syk-Y323F mutant protein was not reduced by co-expression with wild type Cbl (Fig. 5A, third part, lanes 1–5). Furthermore, the levels of CD8-ζ protein were unaffected by HA-Cbl or HA-Cbl-G306E co-expression (bottom part, compare lanes 6–15 with lane 16), and no differences were observed in CD8-ζ levels in cells expressing either wild type Syk or Syk-Y323F (Fig. 5A, bottom part, compare lanes 1–5 with lanes 6–16).

To quantify the extent of the Cbl-induced reduction in the levels of Syk protein and relate this to levels of phosphorylation, densitometric analysis was carried out on the data presented in Fig. 5A (lanes 1–15). The intensity of the Syk band in each lane was normalized to the level of CD8-ζ to adjust for slight differences in protein loading, and the level of Syk phosphorylation or protein in the presence of Cbl was expressed as a ratio to that in the absence of Cbl co-expression. This comparison showed that the Cbl-induced decrease in phosphorytosine signals on Syk and CD8-ζ was accompanied by a parallel reduction in the levels of Syk protein (Fig. 5B).
Since the levels of CD8-z were not affected by HA-Cbl or HA-Cbl-G306E co-expression, the Cbl-dependent reduction in Syk protein levels appears to be selective. Additional experiments using lysates made in SDS-containing lysis buffer showed that the Cbl-induced loss of Syk protein was not a result of its compartmentalization into a Triton X-100-insoluble fraction (data not shown). While a Cbl-dependent decrease in Syk protein levels could have resulted from a PTB domain-dependent effect on Syk message transcription, the absence of any changes in Syk-Y323F levels upon HA-Cbl co-expression or any changes in Syk levels upon HA-Cbl-G306E co-expression argues against this possibility.

**DISCUSSION**

The Syk/ZAP-70 family of tyrosine kinases plays a pivotal role in immune receptor signal transduction and provides a
prominent example of nonreceptor tyrosine kinase-mediated cellular activation (35–37). Therefore, elucidating the mechanisms whereby these tyrosine kinases are regulated is of considerable interest. The Cbl proto-oncoprotein has emerged as a potential negative regulator of receptor and nonreceptor tyrosine kinases, including the Syk/ZAP-70 family (1, 2). The studies reported here provide a structural basis for the physical and functional interactions between Syk and Cbl, and support a critical role for the PTB domain-dependent regulation of tyrosine kinases by the Cbl proto-oncoprotein product.

The current investigation definitively identifies the PTB domain as the major Cbl region involved in the activation-dependent association between Cbl and Syk. This conclusion is based on in vitro binding studies using a GST fusion protein of the Cbl PTB domain and abrogation of Syk binding by a PTB domain-inactivating mutation (G306E) modeled after a loss-of-function mutation identified through genetic screens in the C. elegans Cbl homologue SLI-1 (11) (Fig. 1A). These findings were confirmed in multiple cell lines stimulated either through the B or T cell receptor (Fig. 1, A and B, and data not shown). Filter binding assays demonstrated that the Cbl PTB domain directly binds to Syk in a phosphorylation-dependent manner (Fig. 1C).

Binding studies with GST-Chi-C (which included the remaining portions of Cbl including its RING finger domain, proline-rich region and leucine zipper) clearly indicated that Cbl sequences other than the PTB domain are not sufficient to mediate Cbl/Syk association (Fig. 2A). The Cbl-C fusion protein was functionally competent, as demonstrated by its binding to Grb2 via Grb2 SH3 domains binding to Cbl’s proline-rich region (18, 38, 39). Finally, the Syk SH2 domains did not bind to tyrosine-phosphorylated Cbl, indicating that the Cbl PTB domain-mediated interaction is a predominant mechanism of activation-induced Cbl/Syk association (Fig. 2B). These in vitro studies were complemented by in vivo analyses in COS-7 cells, which showed a requirement for the intact Cbl PTB domain in Cbl/Syk association (Fig. 4B).

In view of a critical role for the Cbl PTB domain in Cbl/Syk association, we wished to delineate the Cbl PTB domain-binding motif on Syk. The consensus Cbl PTB domain-binding motif D(N/D)XpY (7) predicted six potential Cbl PTB domain-binding sites in Syk, three of which (Tyr323, Tyr326, and Tyr340) corresponded to identified autophosphorylation sites (32). Phosphopeptide competition experiments followed by expression of Tyr → Phe mutants of Syk in COS-7 cells demonstrated that TyrP(323) represents a major Cbl PTB domain binding site on Syk (Figs. 3 and 4A). Independently, Deckert et al. (23) have shown that Cbl binding to Tyr316 in porcine Syk (equivalent to Tyr(P)323) is important for Syk/Cbl association in a yeast two-hybrid system (23). Together, these studies strongly indicate that TyrP(323) is a major Cbl PTB domain binding site on Syk. The Syk TyrP(323) corresponds to ZAP-70 Tyr292 (31), the functional role of TyrP(323) in the regulation of Syk (Ref. 15 and this study), and possession of Syk SH2 domains that might interact with Syk/ZAP-70 (5, 42, 43). However, a role for other possible adapters cannot be ruled out at present.

Although TyrP(323) in Syk is analogous to ZAP-70 TyrP(292), a documented negative regulatory phosphorylation site in ZAP-70 (44, 45) and the major Cbl PTB domain binding site on that protein (7), the functional role of TyrP(323) in the regulation of the Syk tyrosine kinase has not been investigated. Therefore, we examined if Cbl’s interaction with TyP(323) was important for negative regulation of Syk. Similar to Cbl’s effect on Syk in RBL-2H3 mast cells (15), co-expression of Syk and Cbl in a COS cell reconstitution system induced a marked and Cbl dose-dependent reduction of phosphoryrosine signals on Syk and CD8+, an in vivo substrate of Syk in transfected COS-7 cells (Fig. 5). Either inactivation of the Cbl PTB domain by G306E mutation or Syk-Y323F mutation led to a complete abrogation of Cbl’s negative regulatory influence on Syk (Fig. 5). Together, these analyses directly demonstrated that the interaction of the Cbl PTB domain with the TyrP(323) motif in autophosphorylated Syk is critical for Cbl-mediated negative regulation of Syk. Thus, recruitment of Cbl to an autophosphorylation site within the SH2-kinase interdomain linker region, which is created upon kinase activation, may provide a shared mechanism of activation-dependent negative regulation of the Syk/ZAP-70 family of tyrosine kinases. It can be speculated that the level of available Cbl may provide a mechanism to determine the extent of Syk/ZAP-70 activation and hence the intensity of downstream signals. Notably, the levels of Cbl mRNA expression are highest in the thymus and in other hematopoietic tissues and cell lines (46), and a recent study found evidence of hypercellularity in the thymus and spleen of Cbl−/− knockout mice (16).

Unexpectedly, careful quantification revealed a drastic loss of the Syk protein in transfected COS-7 cells under conditions where Cbl was able to negatively regulate Syk (wild type Cbl together with wild type Syk) but not under conditions where it...
was unable to induce a negative regulatory effect (wild type Cbl with Syk-Y232F or Cbl-G306E with wild type Syk). The loss of Syk protein upon Cbl co-expression was dose-dependent (Fig. 5B) and was not due to compartmentalization into a detergent-insoluble pool, since the same results were obtained with cell lysates prepared in SDS-containing lysis buffer (data not shown). Since wild type Syk and Syk-Y232F cDNAs differ by a single nucleotide, the loss of Syk protein is unlikely to be due to altered transciption or message stability. It is more likely that interaction with Cbl targets the autophosphorylated Syk for degradation, suggesting a potentially novel pathway for the negative regulation of Syk/ZAP-70 tyrosine kinases. However, it will be important to determine whether Cbl-dependent Syk degradation occurs in lymphoid cells and whether degradation is a primary mode of negative regulation as opposed to a mechanism for the removal of Syk protein rendered inactive through a different mechanism.

Although we did not observe a decrease in CD8-ι or Cbl protein levels under our experimental conditions, the T cell receptor ζ and FceR1 α chains, which serve as ZAP-70/Syk-docking proteins in T cells and RBL-2H3 mast cells, respectively, are known to be ubiquitinated upon immune receptor stimulation (3, 47–49, and Cbl was shown to be reversibly ubiquitinated upon GM-CSF receptor stimulation (50). Interestingly, we have observed an increase in the ligand-induced ubiquitation and degradation of the PDGF-Rα in NIH-3T3 cells that overexpress Cbl (51). However, the possible role of ubiquitination in Cbl-dependent Syk degradation or the nature of the degradation machinery remains to be clarified.

A number of recent findings collectively provide clear evidence that the PTB domain is crucial to Cbl’s negative regulation of tyrosine kinases: this domain is highly conserved during evolution (11–13, 52); it is the site of loss-of-function mutations of the degradation machinery remains to be clarified.}

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Cbl-mediated Negative Regulation of the Syk Tyrosine Kinase: A CRITICAL ROLE FOR Cbl PHOSPHOTYROSINE-BINDING DOMAIN BINDING TO Syk PHOSPHOTYROSINE 323
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