Coordinated Regulation of the Tyrosine Phosphorylation of Cbl by Fyn and Syk Tyrosine Kinases*

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Cross-linking of the T cell antigen receptor (TCR)-CD3 complex induces rapid tyrosine phosphorylation and activation of Src (Lck and Fyn) and Syk (Syk and Zap-70) family protein tyrosine kinases (PTKs) which, in turn, phosphorylate multiple intracellular substrates. Cbl is a prominent PTK substrate suggesting a pivotal role for it in early signal transduction events. However, the regulation of Cbl function and tyrosine phosphorylation in T cells by upstream PTKs remains poorly understood. In the present study, we used genetic and biochemical approaches to demonstrate that Cbl directly interacts with Syk and Fyn via its N-terminal and C-terminal regions, respectively. Tyr-316 of Syk was required for the interaction with Cbl as well as for the maximal tyrosine phosphorylation of Cbl. However, both wild-type Syk and Y316F-mutated Syk phosphorylated equally well the C-terminal fragment of Cbl in vivo, suggesting the existence of an alternative, N terminus-independent mechanism for the Syk-induced tyrosine phosphorylation of Cbl. This mechanism appears to involve Fyn, since, in addition to its association with the C-terminal region of Cbl, Fyn also associated with Syk and enhanced the Syk-induced tyrosine phosphorylation of Cbl. These findings implicate Fyn as an adaptor protein that facilitates the interaction between Syk and Cbl, and suggest that Src and Syk family PTKs coordinately regulate the tyrosine phosphorylation of Cbl.

Binding of antigenic peptides presented by major histocompatibility complex molecules to the T cell receptor (TCR)-CD3 complex induces a rapid increase in the activities of two families of nonreceptor PTKs, i.e. the Src (Fyn and Lck), and Syk (Syk and Zap-70) families (1, 2). Activation of Lck and/or Fyn leads to tyrosine phosphorylation of immunoreceptor tyrosine-based activation motifs present in the intracellular domains of CD3 and ζ subunits, resulting in the subsequent recruitment and activation of Zap-70 and Syk. The activated PTKs in turn propagate activation signals by phosphorylating multiple intracellular proteins, eventually leading to T cell activation, lymphokine production, and proliferation. However, many aspects of the underlying signaling cascades remain unclear.

The c-cbl (Casitas B-lineage lymphoma) protooncogene was originally isolated as a cellular homologue of v-cbl, a part of the transforming gene of the Cas NS-1 murine leukemia retrovirus (3, 4). The 120-kDa product of c-cbl, Cbl, is rapidly phosphorylated on tyrosine residues in response to stimulation of various cell surface receptors, including the TCR (5–7) (reviewed in Liu and Altman (5)), suggesting a critical role for Cbl in signal transduction pathways. The importance of Cbl in intracellular signal transduction is further emphasized by the observation that it associates with several other signaling molecules. Numerous studies have shown that Cbl binds constitutively to Grb2 (6–9). Tyrosine phosphorylation of Cbl results in its inducible association with phosphatidylinositol 3-kinase (7, 8, 10–12), with CrkL (13–20), and with Vav (21). We recently demonstrated an interaction between Cbl and 14-3-3 proteins (22), which depends on serine phosphorylation of Cbl (23).

Reconstitution studies in COS-1 cells showed that Zap-70 phosphorolyses Cbl in an Lck- and/or Fyn-dependent manner (24). Syk has been shown to have a greater intrinsic enzymatic activity than Zap-70 in terms of Cbl tyrosine phosphorylation (25). Syk alone can cause tyrosine phosphorylation of Cbl, which is enhanced by coexpression of Lck (23). Overexpression of Syk in mast cells enhances the FceRI-mediated tyrosine phosphorylation of Cbl (26). The notion that Cbl is phosphorylated by Src and/or Syk family kinases is complemented by the observations that Cbl can physically associate with these PTKs. Both Src family kinases, such as Fyn (6, 8, 24, 27, 28) or Lyn (29), and Syk (26, 27) or Zap-70 (24, 30), have been reported to associate with Cbl. The association of Zap-70 with Cbl is activation-dependent, and is mediated by a phosphotyrosine (Tyr(P))-binding (PTB) domain in the N-terminal region of Cbl (20). A point mutation (G306E) in this domain of c-cbl, which corresponds to a loss-of-function mutation in Slit-1, a Caenorhabditis elegans Cbl homologue (31), disrupts the interaction of Cbl with Zap-70, indicating that the N-terminal portion of Cbl is required for the interaction. However, the C-terminal region of Cbl has also been implicated in the constitutive interaction of Cbl with Syk in mast cells (26).

The interactions of Syk/Zap-70 and Fyn with Cbl strongly suggest that Cbl is involved in Syk/Zap-70- and/or Fyn-dependent signaling pathways which are stimulated by TCR ligation. However, the regulation of Cbl tyrosine phosphorylation by upstream PTKs remains poorly understood. As a first step toward understanding the biological function of Cbl, we have undertaken a study of the interaction of Cbl with Syk and/or Fyn using genetic and biochemical approaches. We demonstrate that Syk binds to the N terminus of Cbl, whereas Fyn binds to its C terminus. We further define a critical tyrosine residue of Syk, Tyr-316, which is required for the direct interaction with, and for the tyrosine phosphorylation of, Cbl. In addition, our results suggest that Fyn can act as an adaptor protein that facilitates the interaction between Cbl and Syk.

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‡ The abbreviations used are: TCR, T cell antigen receptor; GST, glutathione S-transferase; mAb, monoclonal antibody; PTK, protein tyrosine kinase; Tyr(P), phosphotyrosine; PTB, phosphotyrosine binding; IP, immunoprecipitation; HA, hemagglutinin; SD, synthetic dropout.

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and the tyrosine phosphorylation of Cbl, suggesting a coordinated regulation of Cbl tyrosine phosphorylation by Src and Syk family PTKs.

MATERIALS AND METHODS

Antibodies—Polyclonal rabbit anti-Cbl (c-15) and anti-Fyn antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Tyr(P) monoclonal antibody (mAb) 4G10 and anti-glutathione S-transferase (GST) polyclonal antibody were from Upstate Biotechnology (Lake Placid, NY). Anti-hemagglutinin (HA) mAb (12CA5) was from Boehringer Mannheim. An anti-Cd4 (OKT3) and an anti-HIV-derived tag epitope (H902) (32) mAbs were purified from ascetics fluid by protein A-Sepharose affinity chromatography. Horseradish peroxidase-conjugated P(ab)2 fragments of donkey anti-rabbit IgG or sheep anti-mouse IgG were from Amersham Corp.

Plasmids—The pLex and pACT2 vectors (CLONTECH) were used in the yeast two-hybrid system. cDNAs corresponding to full-length Cbl, v-Cbl (Cbl residues 1–365), and the C-terminal portion of Cbl (Cbl-C; amino acid residues 450–906) were fused in-frame to the yeast Gal4 activation domain encoded by a pACT2 vector. The pLex vectors containing Zap-70, Syk, or Syk mutants including SykK395R, Y518F/Y519F, 1–298, 1–212, 213–628, Y341F, and Y345F, and the pACT2 vector containing Vav, were described previously (32). Additional Syk point mutations including Y316F or Y500F were made by site-directed mutagenesis based on the pLex-Syk vector. cDNAs encoding wild-type Syk or Syk Y316F were subcloned into a mammalian vector, pEFneo (33), which has been tagged with an HA epitope. An H902-tagged Syk in pME18S was described (32). Fyn cDNA in pME18S was provided by T. Kawakami. The Fyn cDNA derived from pME18S was subcloned into pLex in an in-frame fashion. cDNAs encoding HA-tagged wild-type Cbl, 70Z, v-Cbl, Cbl-C, and successive C-terminal deletion mutants of HA-tagged 70Z in pEFNeo were reported (34). Cbl-C and its successive N-terminal deletion mutants constructed by using a polymerase chain reaction were subcloned into pEFNeo downstream of an HA902 tag epitope (32).

Yeast Two-hybrid System—The yeast two-hybrid system used in this study was essentially the same as described previously (32). Briefly, the yeast strain L40 was cotransformed with pLex and pACT2 vectors containing appropriate cDNAs and cotransformants were first screened on plates containing synthetic dropout (SD) medium lacking Leu and Trp. Colonies were then picked up and grown on plates with SD medium lacking His, Leu, and Trp or in medium lacking Leu and Trp. The transformants were selected in SD medium lacking His, Leu, and Trp or in medium lacking Leu and Trp. Colonies were then picked up and grown on plates with SD medium lacking Leu, Trp, and His to examine protein-protein interactions. The transformants were also tested for β-galactosidase activity using a filter assay.

Cell Culture and Stimulation—Simian virus 40 T antigen (TAG)-transfected human leukemic Jurkat T cells (Jurkat-TAg) were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics. Cells were resuspended (2 × 10^6/ml) in 0.5 ml of medium, equilibrated at 37 °C for 5 min, and activated with OKT3 (4 µg/ml) for 5 min. Stimulation was terminated by adding 0.5 ml of 2% Nonidet P-40 lysis buffer (2% Nonidet P-40, 40 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 4 mM Na3VO4, 20 µg/ml each of aprotinin and leupeptin). Cells were lysed for 10 min at 4 °C and insoluble materials were removed by centrifugation at 15,000 × g (4 °C for 10 min).

COS-1 cells were cultured in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc.) containing 10% heat-inactivated fetal bovine serum at 37 °C in 5% CO2. For protein expression in Jurkat-TAg and COS-1 cells, cells were transfected with appropriate amount of plasmids (usually 10 µg total) by electroporation as described previously (23).

Expression of GST Fusion Proteins in a Baculovirus System—The cDNAs encoding v-Cbl, or Cbl-C were subcloned into a baculovirus GST fusion vector, pAc-GST (Pharmingen) which, in addition to encoding a GST fusion partner, also encodes a 6× His tag in an in-frame fashion. Recombinant virus carrying these Cbl constructs were generated by cotransfection with wild-type baculovirus DNA into the insect cell line, Sf9, using a Baculogold transfection kit (Pharmingen), were further amplified, and were used for infection of Sf9 cells. The GST-Cbl fusion proteins were then purified by using gluthathione or Ni2⁺-nitrilotriacetic acid affinity columns.

In Vitro Binding Assay and Far Western Blotting—For in vitro binding assays, cell lysates were incubated with 10 µg of GST fusion proteins for 2 h at 4 °C, followed by adding 40 µl of glutathione-Sepharose beads as described previously (35). After 1 h at 4 °C, the binding mixtures were washed extensively in 1× Nonidet P-40 lysis buffer and used for further analysis. The procedure for Far-Western blotting was described previously (23). The GST-Fyn SH2 fusion protein was purchased from Upstate Biotechnology.

Immunoprecipitation and Immunoblotting—Lysates (1 × 10^6 cells) were mixed with antibodies for 2 h, followed by addition of 40 µl of protein A/G Plus-Sepharose beads (Santa Cruz Biotechnology) for an additional 4 h at 4 °C. Immunoprecipitates (IPs) were washed four times with 1× Nonidet P-40 lysis buffer, and boiled in 30 µl 2× Laemmli’s buffer. Samples were subjected to SDS-polyacrylamide gel electrophoresis, 10% gel, analysis and electrotransferred onto polyvinylidene difluoride membranes (Millipore). Membranes were immunoblotted with the indicated primary antibodies (usually 1 µg/ml), followed by horseradish peroxidase-conjugated secondary antibodies. Membranes were washed and visualized with enhanced chemiluminescence detection system (ECL, Amersham Corp.). When necessary, membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS for 1 h at 70 °C with constant agitation, washed, and then reprobed with other antibodies as indicated.

RESULTS

The Interaction of Fyn with Cbl in Yeast—It was previously reported that Fyn associates with Cbl in T cells, and that this interaction is increased upon T cell activation (28). To further define the interaction between Fyn and Cbl, we first used the yeast two-hybrid system. As shown in Fig. 1A, cells transformed with both full-length Cbl or Cbl-C (and Fyn), but not with v-Cbl, exhibited growth in medium lacking Leu, Trp, and His, suggesting that the interaction of Cbl with Fyn is mediated by the C-terminal region of Cbl. As a negative control, no interaction was observed between Fyn and the empty pACT2 vector.

Next, we wished to more precisely dissect the Fyn-binding domain in the C-terminal region of Cbl. Two cDNA fragments encoding the proximal (amino acid residues 450–730) or distal (residues 731–906) portions of Cbl-C were subcloned into the

FIG. 1. Interaction of Fyn with Cbl in yeast. A, yeast (L40) was cotransformed with pLex plasmids containing Fyn or Syk cDNA in combination with empty pACT2 vector or pACT2 vector containing full-length Cbl, the N-terminal region of Cbl (v-Cbl), the C-terminal region of Cbl (Cbl-C), or Vav. The transformants were selected in SD medium lacking His, Leu, and Trp or in medium lacking Leu and Trp. B, determination of the Fyn binding region of Cbl in yeast. Two additional Cbl-C constructs encoding amino acids 450–730 or 731–906 in pACT2 were tested for their interaction with Fyn in yeast as in A.
pACT2 vector. As shown in Fig. 1B, similar to full-length Cbl or Cbl-C, yeast transformed with the proximal fragment, but not the distal fragment, of Cbl-C exhibited growth in the selection medium. This result indicates that the region of Cbl encompassing amino acid residues 450–730 contains the binding site(s) for Fyn.

The Interaction of Fyn with Cbl In Vitro and in Vivo—To further examine the interaction between Fyn and Cbl, we performed in vitro binding assays using GST-Cbl fusion proteins. Cell lysates from unstimulated Jurkat T cells or from anti-CD3 (OKT3)-stimulated cells were incubated with GST alone, GST-Cbl-C, or GST-v-Cbl. The precipitates were then analyzed with an anti-Fyn antibody. As shown in Fig. 2A, Cbl-C, but not v-Cbl or GST alone, bound Fyn in an activation-dependent manner (top panel). As a control, the membrane was reprobed with an anti-Grb2 antibody, showing that Cbl-C, but not v-Cbl, constitutively binds Grb2 (bottom panel), as reported earlier (6–9).

Next, we analyzed the Fyn-Cbl interaction in vivo by cotransfecting COS-1 cells with a Fyn plasmid plus a panel of expression vectors encoding HA-tagged C-terminal deletions of Cbl (23). As shown in Fig. 2B (top panel), both Cbl-C and full-length Cbl (70Z) coimmunoprecipitated with Fyn (lanes 1 and 6). C-terminal deletion mutants encoding amino acid residues 1–450 or 1–655 did not coimmunoprecipitate with Fyn (lanes 2 and 3). A mutant encoding amino acids 1–698 associated weakly with Fyn (lane 4), and a mutant encoding residues 1–730 of Cbl displayed an even stronger interaction with Fyn (lane 4 versus 5). Equivalent levels of Fyn were present in all the samples as revealed by reprobing the membrane with the anti-Fyn antibody (Fig. 2B, middle panel). Similarly, the various Cbl mutants were expressed at equivalent levels (Fig. 2B, bottom panel).

To further map the in vivo interaction, we prepared a series of N-terminal deletion mutants derived from Cbl-C. The Fyn-Cbl interaction was then analyzed in COS-1 cells as described above. Cbl mutants encoding amino acid residues 538–906 or 552–906 coimmunoprecipitated with Fyn to a similar degree as did Cbl-C (Fig. 2C, top panel; lanes 2 and 3 versus lane 1).

Deletion of additional 63 (615–906 mutant) or 146 (698–906 mutant) amino acids markedly reduced the interaction of Cbl with Fyn (lanes 4 and 5, respectively). Both the immunoprecipitated Fyn and the expressed Cbl mutants were expressed at similar levels in the different groups as indicated by immunoblotting the membrane with anti-Fyn or H902 antibodies (Fig. 2C, middle and bottom panels, respectively). These results indicate that the region encompassing amino acid residues 552–614 of Cbl is required for Fyn binding.

Analysis of the Syk-Cbl Interaction in Yeast—Syk was recently found to interact with Cbl in rat basophilic leukemia cells (26) and in B cells (27). To further define the interaction between Cbl and Syk, we first used the yeast two-hybrid system. As shown in Fig. 3A, yeast cotransformed with Syk and v-Cbl, but not Cbl or Cbl-C, grew in SD medium lacking Leu, Trp, and His. No interaction was observed between Zap-70 and any of the three Cbl constructs. As a positive control, Syk, but not Zap-70, interacted with Vav (32). This result indicates that Cbl interacts with Syk in yeast via its N-terminal region.

Next, we expanded our studies to map the Cbl-binding site(s) in Syk using the yeast two-hybrid system. As shown in Fig. 3B, v-Cbl did not interact with the Syk constructs including a kinase-inactive Syk point mutant (K395R), a kinase-deficient deletion mutant that contains the N-terminal tandem SH2 domains (Syk 1–298), Syk Y518F/Y519F in which the two regulatory autophosphorylation sites have been mutated, and Syk 1–212 which lacks the kinase domain and the second SH2 domain. The Syk 213–628 mutant which lacks both SH2 domains but is catalytically active, still associated with v-Cbl. These results indicate that the kinase activity and/or the linker/kinase domains of Syk is required for the interaction with Cbl.

Of the four tyrosine-mutated Syk proteins, only the Y316F mutant did not interact with v-Cbl (Fig. 3B). Of note, the Y341F and Y345F mutations, which mediate the association of Syk with Vav (32), or Y500F, had no discernible effect on the interaction with v-Cbl. The specificity of the Y316F mutation is indicated by the finding that it did not disrupt the interaction.
of Syk with Vav under the same conditions (Fig. 3C).

The Interaction of Syk with Cbl in Vitro and in Vivo—To establish the physiological relevance of the Cbl-Syk interaction, it was further explored by an in vitro binding assay using GST-Cbl fusion proteins. Jurkat-TAg cells were transiently transfected with plasmids encoding HA-tagged wild-type or Y316F-mutated Syk. Lysates from unstimulated or anti-CD3-stimulated cells were incubated with GST alone, GST-v-Cbl, or GST-Cbl-C, and the precipitates were probed with an anti-HA antibody. As shown in Fig. 4A, GST-v-Cbl displayed activation-dependent interaction with wild-type Syk but not with Syk Y316F (lane 6 versus 8). A weak but consistent interaction was observed between GST-Cbl-C and wild-type Syk in OKT3-stimulated cells (lane 10), but not with Syk Y316F (lanes 11 and 12). The expression levels of Syk or Syk Y316F in the lysates were similar as detected by immunoblotting cell lysates with the anti-HA antibody (lanes 13 and 14). This result indicates that Tyr-316 of Syk is required for maximal interaction with Cbl. However, the weak interaction of Cbl-C with Syk suggests an alternative, additional mechanism independent of the Cbl N-terminal domain, which mediates (directly or indirectly) an association between Cbl and Syk.

To further analyze the interaction between Syk and Cbl, we cotransfected COS-1 cells with plasmids encoding HA-tagged Syk or Syk Y316F and Cbl. Cell lysates were immunoprecipitated with an anti-Cbl antibody and the IPs were analyzed with an anti-Tyr(P) antibody. As shown in Fig. 4B (top panel), a Cbl-associated phosphoprotein comigrating with authentic Syk was detected in Cbl immunoprecipitates from cells transfected with wild-type Syk (lane 2). A weaker but consistent interaction between Syk Y316F and Cbl was also observed under similar conditions (lane 3). The cell lysates were analyzed in parallel by anti-Tyr(P) immunoblotting, showing that similar levels of Syk proteins were expressed (lanes 5 and 6). These results confirm our previous observations indicating that the Tyr-316 in Syk contributes to the in vivo interaction of Syk with Cbl. In addition, the weak interaction between Syk Y316F and Cbl observed in vivo suggests, again, an alternative mechanism for the Syk-Cbl interaction.

Regulation of Tyrosine Phosphorylation of Cbl by Fyn and Syk—The interaction of Syk and Fyn with the N- or C-terminal regions of Cbl, respectively, suggested that these two PTKs coordinately regulate the tyrosine phosphorylation of Cbl. This is consistent with previous studies demonstrating that both Fyn and Syk can phosphorylate Cbl in different cells (23–26). Next, we addressed the role of Syk and Fyn in the tyrosine phosphorylation of Cbl. COS-1 cells were cotransfected with a Cbl plasmid and plasmids encoding wild-type Syk, Syk Y316F, or Fyn. When lysates from these cells were immunoblotted with an anti-Tyr(P) antibody, coexpressed Syk was found to induce tyrosine phosphorylation of Cbl (Fig. 5A, top panel; lane 2). However, the Syk Y316F mutant caused a significantly lower tyrosine phosphorylation of Cbl (lane 3). This did not reflect lower enzymatic activity of the Y316F mutant since this mutant was phosphorylated on tyrosine to at least the same extent as wild-type Syk (lane 3 versus 2). In contrast, coexpression of Fyn did not cause a detectable increase in the Tyr(P) content of Cbl as compared with cells transfected with Cbl alone (lane 4 versus 1). The same immunoblot revealed the proper expression of Syk, Syk Y316F, or Fyn in the transfected cells. Similar reduced phosphorylation of Cbl by Syk Y316F was observed when Cbl IPs from the cells were probed with an anti-Tyr(P) antibody (Fig. 5A, middle panel). Anti-Cbl immunoblot of Cbl IPs from the same cells revealed similar expression levels of Cbl in the different groups (bottom panel). These results suggest that the putative Cbl-binding site in Syk, Tyr-316, is required for optimal tyrosine phosphorylation of Cbl.

The failure of Fyn to induce tyrosine phosphorylation of Cbl in COS-1 cells suggested that Fyn may play an indirect role in this event. To test this hypothesis, we performed additional
experiments by cotransfecting COS-1 cells with the Cbl plasmid and plasmids encoding Syk, Y316F, or Fyn, either individually or in different combinations. The tyrosine phosphorylation of Cbl in cells cotransfected with any single PTK was essentially the same as before (compare top panels, lanes 1–4, in Fig. 5, A versus B). By contrast, coexpression of Cbl with Syk or Syk Y316F in the presence of Fyn increased the Tyr(P) content of Cbl (Fig. 5B, top panel; lanes 5 and 6). Of note, under these conditions, no difference in the tyrosine phosphorylation of Cbl was observed between cells expressing wild-type versus Y316F Syk. Thus, Fyn coexpression appears to compensate for the relative inefficiency of Syk Y316F in phosphorylating Cbl. The immunoprecipitated Cbl was expressed at similar levels in all the groups (Fig. 5B, bottom panel). These results provide evidence that Fyn acts in a primarily indirect manner to promote the tyrosine phosphorylation of Cbl.

The respective interactions of Fyn and Syk with Cbl-C or v-Cbl, the Fyn-enhanced tyrosine phosphorylation of Cbl by Syk or Syk Y316F, and the inability of Fyn alone to efficiently phosphorylate Cbl, suggested that Fyn may act as an adaptor to facilitate the phosphorylation of Cbl by Syk. To test this hypothesis, COS-1 cells were cotransfected with a plasmid containing H902-tagged Cbl-C and Syk, Y316F, or Fyn, either individually or in combination. Cbl-C was used in this experiment for two reasons. First, an earlier report indicated that the tyrosine phosphorylation of Cbl occurs exclusively in its C-terminal region (26); and, second, Cbl-C lacks the N-terminal region of the protein, thereby excluding any potential contribution of the interaction between this region and Tyr-316 of Syk to the tyrosine phosphorylation of Cbl. Analysis of Cbl-C (H902 anti-tag antibody) IPs with an anti-Tyr(P) antibody revealed, first, that Cbl was not phosphorylated on tyrosine in cells lacking a coexpressed PTK (Fig. 6A, top panel; lane 1). Syk or Syk Y316F coexpression induced the phosphorylation of Cbl-C on tyrosine to a similar degree (lane 2 versus 3), in contrast to the deficient phosphorylation of full-length Cbl by Syk Y316F (Fig. 5). Similar to full-length Cbl, coexpression of Fyn caused only a weak but detectable tyrosine phosphorylation of Cbl-C (lane 4), but coexpression of Syk or Syk Y316F with Fyn increased the Tyr(P) content of Cbl-C to a similar degree by comparison with cells expressing Syk as the only PTK (lanes 5 and 6 versus lanes 3 and 4). Reprobing the membrane with an anti-Cbl antibody revealed equivalent expression levels of immunoprecipitated Cbl-C in all the samples (Fig. 6A, bottom panel). As additional controls, cell lysates were also immunoblotted with an anti-Fyn or an anti-HA antibody, revealing the expression of Fyn, HA-Syk, or HA-Syk Y316F, in the appropriate groups (Fig. 6B). The results indicate that, in the absence of the N-terminal region of Cbl, both wild-type Syk and Syk Y316F exhibit the same kinase activity toward Cbl-C. This finding provides further support for the notion that an alternative mechanism exists for the Syk-induced tyrosine phosphorylation of Cbl. This mechanism does not require an interaction between Syk and the N-terminal region of Cbl, but is enhanced by Fyn.

To establish the physiological relevance of the tyrosine phosphorylation of Cbl observed in COS-1 cells, we performed similar experiments in unstimulated or anti-CD3-activated Jurkat-TAg cells which were transfected with plasmids encoding H902-tagged intact Cbl-C or its N-terminal deletion mutants. H902 IPs from these cells were then analyzed with an anti-Tyr(P) antibody. As shown in Fig. 7 (top panel), OKT3 stimulation induced the tyrosine phosphorylation of Cbl-C, Cbl 538–
906, and Cbl 552–906 mutants to a similar degree (lanes 2, 4, and 6). In contrast, the truncated Cbl-C proteins corresponding to residues 615–906 or 698–906 displayed a marked decrease in their OKT3-induced tyrosine phosphorylation (lanes 8 and 10, respectively). The membrane was reprobed with an anti-Cbl antibody, showing that similar amounts of the different Cbl proteins were present in the samples (Fig. 8). Of interest, the phosphorylation pattern of the Cbl-C mutants coincided precisely with their ability to coimmunoprecipitate with Fyn. Thus, deletion of Cbl residues 552–614 reduced in parallel the association of Cbl with Fyn (Fig. 2C) and its phosphorylation in activated T cells (Fig. 7). These findings suggest that interaction with Fyn is required for the optimal tyrosine phosphorylation of Cbl.

Interaction between Fyn and Syk—The above findings lend support for the notion that Fyn, via its association with Cbl, functions as an adaptor to facilitate the interaction between Syk and Cbl. Such an adaptor function would imply that Fyn also associates (directly or indirectly) with Syk. To address this, we analyzed potential interactions between Fyn and Syk both in vitro and in vivo. A GST-Fyn SH2 fusion protein directly bound to Syk as demonstrated by Far-Western blotting of cell lysates from Jurkat-TAg cells transfected with H902-tagged Syk or with an empty vector (pME18S) (Fig. 8A, top panel). Reprobing the same membrane with an anti-Tyr(P) antibody (middle panel) and again with H902 (bottom panel) B and C, interaction of Fyn with Syk in vivo. B, COS-1 cells were transfected with empty pEFneo vector, or with pEF encoding HA-Syk, Fyn, or both. Lysates were immunoblotted with anti-tyr(P) antibody. C, lysates from B were immunoprecipitated with an anti-Fyn antibody. One half of each IP was immunoblotted with an anti-Tyr(P) antibody (top panel), and the remaining IPs were immunoblotted with an anti-HA antibody (middle panel), and then reprobed with an anti-HA antibody (bottom panel). The positions of the overexpressed proteins are indicated by the arrows. Molecular weight markers are shown on the left.
DISCUSSION

Cbl is rapidly phosphorylated on tyrosine upon cross-linking of the TCR-CD3 complex, and also associates with Fyn and the Syk family PTKs, Syk and Zap-70 (reviewed in Liu and Altman (5)). Understanding how the tyrosine phosphorylation of Cbl is regulated by these PTKs is critical for the elucidation of the biological function of Cbl in TCR-mediated signal transduction. Previous studies which have addressed the functional and physical interactions between Cbl and Fyn or Zap-70/Syk (5, 6, 24–28, 30) have, on occasion, led to apparently contradictory findings regarding the relative contribution of Fyn versus Zap-70/Syk to the tyrosine phosphorylation of Cbl or the role of distinct regions of Cbl in binding to these kinases. In the current study, we mapped the interactions between Cbl and Fyn or Syk by using genetic and biochemical approaches. We found that Syk and Fyn interact directly with the N-terminal and C-terminal regions of Cbl, respectively. Tyr-316 of Syk was required both for the interaction with Cbl and for its optimal tyrosine phosphorylation in activated T cells. In addition, evidence was provided that Fyn plays primarily an indirect role by interacting with both Cbl and Syk, and by enhancing Syk-mediated phosphorylation of Cbl. Taken together, these results suggest a coordinated mechanism through which Src- and Syk-family kinases interact to promote the phosphorylation of Cbl, thereby regulating its adaptor function.

Previous studies have shown that the N-terminal region of Cbl (or v-Cbl) interacts with Zap-70 in an activation-dependent manner (30). This implicated the existence of an N-terminal PTB-like domain in Cbl which binds to Tyr(P) residue(s) in Zap-70. The basis for the interaction of Syk with Cbl is less clear, however. Using the yeast two-hybrid system, we detected an interaction of the N-terminal region of Cbl with Syk (but not with Zap-70). The reported lack of interaction between Syk and the N-terminal region of Cbl (26) most likely stems from the fact that the non-interacting N-terminal construct used in that study (residues 1–480) includes the zinc Ring finger of Cbl, whereas our construct (v-Cbl; residues 1–365) does not. Presence of the Ring finger was shown to suppress the transforming activity of v-Cbl (36) and to inhibit its interaction with the activated epidermal growth factor receptor (37), and may similarly prevent the interaction between the PTB-like domain of Cbl and Syk.

The inability of Zap-70 to interact with Cbl in yeast reflects the fact that Zap-70 is inactive in this system (32). Indeed, the interaction between Syk and Cbl in yeast is dependent on intact catalytic activity and the kinase domain of Syk. Significantly, we demonstrated that mutation of Tyr-316 in Syk to phenylalanine disrupted its interaction with v-Cbl. This effect was specific since the same mutation did not affect the interaction between Syk and Cbl-C (residues 1–480) includes the zinc Ring finger of Cbl, whereas our construct (v-Cbl; residues 1–365) does not. Presumably, the Ring finger was shown to suppress the transforming activity of v-Cbl (36) and to inhibit its interaction with the activated epidermal growth factor receptor (37), and may similarly prevent the interaction between the PTB-like domain of Cbl and Syk.

The biological function of Cbl is still unclear. Several studies documented that mammalian Cbl or its C. elegans (Sli-1) and Drosophila (ν-Cbl) homologues act as a negative regulators of PTK-mediated signaling pathways (31, 39–42). However, the mechanisms underlying the observed negative effects in different cell systems appear to be distinct. While the original finding in C. elegans suggested that Sli-1 is a negative regulator of Ras-dependent signals (31), other studies demonstrated that, in mammalian cells, Cbl is a negative regulator of the JAK-STAT (but not Ras) signaling pathway (41), or of Syk (39). The latter study also demonstrated that inhibition of Syk-mediated signals by Cbl depends on physical association between the two proteins, and that the proline-rich domain of Cbl was essential for this inhibition (39). However, another recent report demonstrated that a Cbl-mediated CrkL-C3G-Rap1 signaling pathway is responsible for the defect in IL-2 production in anergic T cells (42). It should be noted, however, that neither Sli-1 nor ν-Cbl, nor the Cbl region required for the inhibition of Syk, contains the CrkL-binding sites which are located in a distal C-terminal region of Cbl (16, 18). On the other hand, Cbl was also reported to be a positive regulator in a Src-mediated signaling pathway leading to bone resorption (43). Clearly, additional studies are needed to elucidate the role of Cbl in intracellular signaling pathways. The current findings, as well as future studies on the biological effects of Cbl mutants deficient in their interaction with PTKs and other binding proteins, provide a framework for understanding the functions of Cbl.

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