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The Linker Phosphorylation Site Tyr^{292} Mediates the Negative Regulatory Effect of Cbl on ZAP-70 in T Cells

Navin Rao,* Mark L. Lupher, Jr., Satoshi Ota,* Kris A. Reedquist,3 Brian J. Druker,† and Hamid Band4*

The protooncoprotein product Cbl has emerged as a negative regulator of tyrosine kinases. We have shown previously that Cbl binds to ZAP-70 through its N-terminal tyrosine kinase binding (TKB) domain. In this study, we demonstrate that overexpression of Cbl in Jurkat T cells decreases the TCR-induced phosphorylation of ZAP-70 and other cellular phosphoproteins. Coexpression of Cbl with ZAP-70 in COS cells reproduced the Cbl-induced reduction in the level of phosphorylated ZAP-70. The effect of Cbl was eliminated by the TKB-inactivating G306E mutation in Cbl as well as by a phenylalanine mutation of Tyr^{292} within the TKB domain binding site on ZAP-70. Notably, the oncogenic Cbl-70Z/3 mutant associated with ZAP-70, but did not reduce the levels of phosphorylated ZAP-70. Overexpression of Cbl, but not Cbl-G306E, in Jurkat T cells led to a decrease in the TCR-induced NF-AT luciferase reporter activity. Overexpression of the TKB domain itself, but not its G306E mutant, functioned in a dominant-negative manner and led to an increase in NF-AT reporter activity. Cbl-70Z/3-overexpressing cells exhibited an increase in both basal and TCR-induced NF-AT luciferase reporter activity, and this trend was reversed by the G306E mutation. Finally, by reconstituting a ZAP-70-deficient Jurkat T cell line, p116, we demonstrate that wild-type ZAP-70 is susceptible to the negative regulatory effect of Cbl, whereas the ZAP-70-Y292F mutant is resistant. Together, our results establish that the linker phosphorylation site Tyr^{292} mediates the negative regulatory effect of Cbl on ZAP-70 in T cells. The Journal of Immunology, 2000, 164: 4616–4626.

Cbol, the 120-kDa product of the c-cbl protooncopogene, is a prominent component of tyrosine kinase signaling cascades downstream of activated cell surface receptors (1–3). These include receptor protein tyrosine kinases, such as the platelet-derived growth factor receptor (PDGFR)^5 and the epidermal growth factor receptor (EGFR), as well as receptors that non-covalently associate with protein tyrosine kinases, such as the TCR, the B cell receptor, Fc receptors, and cytokine receptors (1–6).

Cbl lacks intrinsic enzymatic activity, but interacts with a variety of Src homology 2 (SH2) and SH3 domain-containing proteins. For example, the interactions between the proline-rich region of Cbl (residues 481–690) and SH3 domains mediate the constitutive association of Cbl with Src family kinases, such as Fyn and Lck, and the adaptor proteins Nck and Grb2 (1–3). Documented tyrosine phosphorylation sites within the C-terminal portion of Cbl mediate its TCR activation-dependent association with SH2 domain-containing proteins such as Vav (Tyr^{770}), the p85 subunit of phosphatidylinositol-3 kinase (Tyr^{341}), and the Crk adapter proteins (Tyr^{774}) (7, 8). These features suggest that one role for Cbl may be to serve as a scaffold to assemble signaling protein complexes (1, 2, 4–6). However, the C-terminal region that mediates these protein-protein interactions is least conserved through evolution: both mammalian EGFR and Caenorhabditis elegans (SLI-1) Cbl homologues as well as the recently cloned third mammalian Cbl-family gene product, Cbl-3, lack most of these C-terminal motifs (1–3, 9). In contrast, the N-terminal domains of Cbl are highly conserved through evolution, suggesting their critical functional role(s): these domains include a RING finger domain and the N-terminal region (Cbl-N) corresponding to sequences retained in the v-cbl oncogene. Recent results have demonstrated that Cbl-N functions as a tyrosine kinase binding (TKB) domain via a direct interaction with a number of autophosphorylated tyrosine kinases, including the lymphocyte tyrosine kinases ZAP-70 and Syk, and the receptor tyrosine kinases PDGFR αβ and EGFR (10–16). The crystal structure of the TKB domain of Cbl in complex with a phosphopeptide, representing its binding site in ZAP-70, revealed that this domain is composed of a four-helical domain, an EF hand, and a variant SH2 domain, with all three of these modules required for phosphopeptide binding (17).

Recent genetic studies support a novel role of Cbl as an evolutionarily conserved negative regulator of tyrosine kinases (1, 2, 5, 6, 18). The C. elegans Cbl homologue, SLI-1, was identified during a screen for negative regulators of LET-23, a homologue of mammalian EGFR. The loss of function mutations in SLI-1, including a single substitution G315E, mapped to the conserved TKB domain (19). The D. melanogaster Cbl homologue D-Cbl

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5 Abbreviations used in this paper: PDGFR, platelet-derived growth factor receptor; Cbl-N, N-terminal transforming region of Cbl; EGFR, epidermal growth factor receptor; HA, hemagglutinin; MAP, mitogen-activated protein; PVDF, polyvinylidene difluoride; SH, Src homology; TKB, tyrosine kinase binding.
was shown to negatively regulate R7 photoreceptor development, a process mediated by an EGFR homologue (20, 21). Interestingly, ablation of c-cbl in mice leads to hyperproliferation in several lymphoid organs, such as the thymus and spleen, and increased ductal branching in the mammary fat pads (22). Furthermore, Cbl deficiency promoted the positive selection of CD4+ T cells when examined in the context of an anti-H2-specific transgene (23).

Expression of wild-type or oncogenic mutant Cbl proteins in mammalian cells has further demonstrated the role of Cbl as a negative regulator of EGFR and PDGFR tyrosine kinases (10, 24–26). In addition, analyses of Cbl-deficient macrophages have demonstrated that Cbl regulates ubiquitination and subsequent endocytosis of the CSF-1 receptor, another receptor tyrosine kinase (27). Notably, overexpression of Cbl in a rat basophilic leukemia cell line was shown to reduce the FceRI-mediated degranulation as well as Syk autophosphorylation and kinase activity (28). A Cbl-induced reduction of the kinase-active pool of Syk was also demonstrated in a reconstituted COS cell system (14). Importantly, the Cbl-TKB domain binding sites on ZAP-70 (13) and Syk (14, 15) correspond to a negative regulatory phosphorylation site within the linker region between the SH2 and kinase domains (ZAP-70-Y292 and Syk-Y323) (29–32). Notably, mutation of these residues leads to enhanced signaling through ZAP-70 and Syk in vivo, yet ZAP-70-Y292F did not exhibit any increase in kinase activity (31, 33). These results suggested that the negative regulatory phosphorylation site in the linker region recruits a negative regulator to ZAP-70 and Syk. Given the specific interaction of Cbl with these sites, Cbl represents a candidate negative regulator to ZAP-70 and Syk. More recently, analyses of Cbl-deficient T cells and reconstitution in COS cells and ZAP-70-deficient Jurkat cells (American Type Culture Collection, Manassas, VA) were maintained as previously described (14).

Materials and Methods

Cells and Abs

Jurkat-JMC (a ZAP-70 and Syk-expressing Jurkat T cell line) and its SV40 T Ag-expressing derivative, JMC-T, were maintained as described (12). The Jurkat-derived ZAP-70/Syk-deficient p116 cell line was kindly provided by Dr. R. T. Abraham (Department of Immunology, Mayo Clinic, Rochester, NY), and was maintained as described (35). The retrovirally transfected clonal derivatives of Jurkat JMC (JMC-HA-Cbl), overexpressing HA-tagged Cbl protein, were maintained as described (36). COS-7 cells (American Type Culture Collection, Manassas, VA) were maintained as previously described (14).

The Abs used in this work were: mouse mAb 4G10 (anti-Tyr(P); IgG2a) (37), mAb SPV-T3b (anti-CD3ε; IgG2a) (38), mAb 12CA5 (anti-influenza hemagglutinin (HA) epitope tag; IgG2b) (39), mAb anti-Syk (IgG2a; Santa Cruz Biotechnology, Santa Cruz, CA), mAb anti-ZAP-70 (IgG2a; Transduction Laboratories, Lexington, KY), mAb anti-β-chain (IgG1; Santa Cruz Biotechnology), mAb W6/32 (anti-class I HLA; IgG2a) (40), rabbit polyclonal anti-phospho MAP kinase (New England Biolabs, Beverly, MA), rabbit polyclonal anti-MAP kinase (New England Biolabs), and PE-conjugated goat anti-mouse Ig (Jackson ImmunoResearch, West Grove, PA).

cDNAs and site-directed mutagenesis

The Lck mammalian expression vectors pdKCR-Lck and pdKCR-Lck-505F (41) were kindly provided by Dr. Y. Minami (Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan). The pSRneoCD8α plasmid encoding a CD8α transmembrane domain and TCR-ζ chain (cytoplasmic region) chimera has been previously described (13). The pAlterMAX-ZAP-70, pAlterMAX-ZAP-70-Y292F, pAlterMAX-HA-Cbl, and pAlterMAX-HA-Cbl-G306E have also been described (14). pAlterMAX-HA-Cbl-70Z/3 was derived by subcloning the appropriate fragment of pSRneo-HA-Cbl-70Z/3 in pAlterMAX using KpnI and SalI restriction sites. pAlterMAX-HA-Cbl-70Z/3-G306E was generated by site-directed mutagenesis of pAlterMAX-HA-Cbl-70Z/3 using the Altered Sites-II Mammalian Mutagenesis System (Promega, Madison, WI), according to the manufacturer’s protocol. The mutagenic oligonucleotide used was 5’-AGT AAC ATA CTC AAT AGC CCA-3’. All constructs were verified by automated DNA sequencing. All Cbl constructs encode N-terminally HA-tagged Cbl proteins. The 70Z/3 Cbl encodes a protein with deletion of residues 366–382 and corresponds to an oncogenic Cbl mutant derived from the murine B cell lymphoma cell line 70Z/3 (42). The NF-AT luciferase reporter plasmid, designated NF-AT-Luc, was a generous gift from Dr. A. Rao (Center for Blood Research, Harvard Medical School, Boston, MA) and contains three repeats of the NF-AT binding sites from the distal promoter of the murine IL-2 gene linked to the firefly luciferase gene (43).

NF-AT luciferase assay

JMC-T or p116 cells were transfected with the indicated amounts of NF-AT luciferase construct and Cbl or ZAP-70 plasmids by electroporation, as previously described (31). The cells were cultured for 12–24 h and then seeded in replicates of five for each stimulation condition (2 × 10^6 cells/well). The cells were stimulated for 6–8 h at 37°C with media alone, anti-CD3 (CD3; 1/2000 dilution of SpVt3b ascites; determined to be optimal in titration experiments), or 50 ng/ml PMA plus 1 μg/ml phorbol myristate acetate (Sigma, St. Louis, MO). Cells were lysed using Cell Culture Lysis Reagent (Promega), and cleared lysates were normalized by the Bradford assay. Equal amount of each lysate was analyzed for luciferase activity using a Monolight 3010C luminometer (Analytical Bioluminescence Laboratory, San Diego, CA) and Luciferin Reagent (Promega), according to the manufacturer’s protocol. The means of five replicates of luciferase units for unstimulated (medium alone) or anti-CD3-stimulated (CD3) cells was expressed as a percentage of the mean luciferase units for PMA plus ionomycin stimulation with the combined SE of both means used in the calculation. For biochemical analysis, an aliquot of the same cells that were used for the NF-AT luciferase assay was cultured for a total of 48 h before cell lysates were prepared.

Transient expression in COS cells

COS-7 cells were plated overnight and transfected for 6 h using Lipofectamine (Life Technologies, Gaithersburg, MD) in OPTI-MEM medium, according to the manufacturer’s protocol. The amounts of each plasmid used for transfection are indicated in the appropriate figure legend. The total amount of DNA for each transfection was held constant with pAlterMAX vector. Cells were lysed 48 h posttransfection in lysis buffer containing 0.5% Triton X-100, 50 mM Tris (pH 7.5), 150 mM sodium chloride, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 μg/ml each of leupeptin, pepstatin A, antipain, and chymostatin.

Surface TCR analysis of Jurkat T cell transfectants

For surface TCR staining, cells were washed twice with ice-cold PBS containing 2% FCS and incubated with the isotype-matched negative control mAb (anti-Syk), an anti-class I HLA mAb W6/32 (positive control), and anti-CD3 mAb (SPV-T3b). Cells were then washed and stained with a PE-conjugated goat anti-mouse secondary Ab. Flow cytometry, data collection, and analysis were performed on a FACSort machine using CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Cell lysis, immunoprecipitations, gel electrophoresis, and immunoblotting

Jurkat T cell transfectants were either left unstimulated or stimulated with anti-CD3 mAb (SPV-T3b) at 37°C and then lysed in the buffer described above (12). Immunoprecipitations from aliquots of COS-7 or Jurkat cell lysates were performed as described (12). Immunoprecipitated proteins or
whole cell lysates were resolved by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA), and immunoblotted with the indicated Abs, essentially as described (13). Protein A-HRP (Cappel-Organon Technika, Durham, NC) was used as a secondary reagent for blotting. Blots were visualized using enhanced chemiluminescence (ECL) and exposed to XBI film (New England Nuclear, Ltd. Sciences, Boston, MA). Blots were stripped and reprobed as described (14). Images of blots were generated by direct scanning of films using a Hewlett-Packard (Palo Alto, CA) ScanJet 4c scanner and Corel Draw version 6 software. Densitometry was conducted on directly scanned images using ScionImage for Windows software.

Results

The phenotype of the Cbl-deficient mice (22, 23) together with the identification of the Cbl TKB domain binding site on ZAP-70 as the negative regulatory phosphorylation site (12, 13) strongly suggested that Cbl down-regulates proximal events in the TCR signaling cascade through negative regulation of ZAP-70. To directly assess whether this is the case and to establish that Cbl-dependent negative regulation of ZAP-70 in T cells was mediated via the negative regulatory phosphorylation site in ZAP-70, we utilized a transfection approach, as described below.

Overexpression of Cbl in Jurkat T cells reduces the level of TCR-induced tyrosine phosphorylation and MAP kinase activation

To assess the effect of Cbl on proximal events triggered by TCR stimulation, we utilized Jurkat T cells overexpressing HA-tagged Cbl as a result of retroviral transfection (36). Three HA-Cbl-overexpressing clones were chosen for these analyses. Each of these clones, C12, D3, and E10, expressed ~10-fold higher levels of Cbl compared with the parental Jurkat JMC cell line (Fig. 1A). Surface staining of the CD3 complex revealed that all three clones expressed cell surface TCR levels comparable with those on the parental Jurkat JMC cell line (Fig. 1B). Staining with an Vß8-specific Ab also revealed comparable levels of surface TCR on Cbl-transfected clones vs parental cells (data not shown).

To analyze the effect of Cbl overexpression on TCR-induced cellular activation, the parental Jurkat JMC cell line and the Cbl-overexpressing clones were stimulated over a 30-min time period with an anti-CD3 Ab and then lysed. The lysates were blotted with an anti-phosphotyrosine Ab to assess the level of tyrosine phosphorylation of various cellular proteins. The Cbl-overexpressing clones exhibited lower levels of anti-CD3-induced phosphorylation on various cellular phosphoproteins when compared with the parental cells (Fig. 2A). Essentially all of the major substrates of tyrosine phosphorylation, such as Syk, ZAP-70, LAT, and ζ-chain, showed reduced levels of phosphorylation in the Cbl-overexpressing clones, with the exception of the 120-kDa polypeptide, which corresponds to overexpressed Cbl.

To analyze the levels of autophosphorylated ZAP-70, cell lysates were immunoprecipitated with an anti-ZAP-70 Ab and then blotted with an anti-phosphotyrosine Ab (Fig. 2B, upper panel). In cells overexpressing Cbl, reduced levels of autophosphorylated ZAP-70 were detected at all time points (Fig. 2B). Very low phosphotyrosine signal was detected in lysates of Cbl transfectants at 30 min poststimulation (Fig. 2B, lanes 8, 12, and 16) when compared with the parental cell line in which signal was still detectable (lane 4). Immunoblotting of whole cell lysates with an anti-ZAP-70 Ab showed relatively equivalent levels of ZAP-70 protein at various time points poststimulation (Fig. 2B, lower panel). Thus, Cbl overexpression in Jurkat T cells appears to lead to selective reduction in the phosphorylated pool of ZAP-70.

In view of the reduced levels of TCR-induced autophosphorylated ZAP-70 and other cell phosphoproteins in Cbl-overexpressing Jurkat T cells, we wished to examine signaling events further downstream of TCR activation. For this purpose, we chose to analyze the levels of phospho-MAP kinases p42 and p44, which reflect the activation of the Ras/Raf/MAP kinase signaling cascade, a key tyrosine kinase-dependent downstream signaling pathway in T cells (44). Parental Jurkat cells and a Cbl-overexpressing clone (C12) were stimulated through the TCR, and the cell lysates were immunoblotted with a phospho-MAP kinase-specific Ab (Fig. 2C). A substantial reduction in the duration of MAP kinase activation was observed in Cbl-overexpressing cells compared with the parental Jurkat JMC cell line. An aliquot of the same cell lysates was blotted with a MAP kinase Ab to demonstrate equivalent levels of MAP kinase. Thus, two independent assay systems provided evidence that Cbl overexpression reduces the level of activation events triggered by stimulation of the TCR in a T cell system.

Reconstitution in COS cells reveals Cbl-mediated negative regulation of ZAP-70 and an essential role for the Cbl TKB domain and ZAP-70 Tyr292

Based on the above findings and the fact that Cbl interacts directly with ZAP-70 in an activation-dependent manner, we wished to determine whether Cbl-dependent negative regulation was due to a direct effect on ZAP-70, and whether the interaction between the Cbl TKB domain and ZAP-70 Tyr292 was required for this negative regulation. For this purpose, we first utilized a COS cell reconstitution system in which cotransfection of ZAP-70 with a Src family kinase (Lck or Lck 505F in our analyses) and an immunoreceptor tyrosine-based activation motif-bearing docking chain (a...
with Cbl (second panel, lane 4). Notably, the Cbl-70ZJ3 mutant also associated with ZAP-70, in fact at a higher level compared with wild-type Cbl (second panel, compare lanes 1 and 3), and this interaction was also abrogated by the Y292F mutation in ZAP-70 (second panel, lane 2). In each case, immunoprecipitation of HA-tagged Cbl proteins was confirmed by anti-HA immunoblotting (Fig. 3, first panel), and equivalent levels of ZAP-70 expression were confirmed by immunoblotting of whole cell lysate with anti-ZAP-70 Ab (Fig. 3, third panel). Taken together, these results indicated that Cbl associates with ZAP-70 in a TKB-dependent manner, and that the association of both wild-type Cbl as well as the Cbl-70ZJ3 mutant protein requires an intact Tyr292 on ZAP-70.

In view of the requirement for Tyr292 to recruit Cbl (Fig. 3), a potential negative regulator, and the hyperresponsive phenotype of ZAP-70-Y292F or ZAP-70 mutants with a deletion of the entire SH2-kinase linker when expressed in lymphocytes (33, 45), we hypothesized that Cbl’s association with phosphorylated Tyr292 would lead to a negative regulatory effect on ZAP-70. To directly test this idea, we examined the effect of Cbl coexpression on the autophosphorylation of ZAP-70. COS cells were cotransfected with cDNA constructs encoding ZAP-70 or the ZAP-70-Y292F mutant together with CD8-ζ and the Src family kinase Lck. In addition, graded amounts of Cbl or its G306E mutant were coexpressed, and whole cell lysates were analyzed by anti-phosphotyrosine, anti-HA, anti-ZAP-70, and anti-ζ immunoblotting (Fig. 4). As previously observed, coexpression of Lck in this system was essential for tyrosine phosphorylation of CD8-ζ and ZAP-70 (Fig. 4A, top panel, lane 15).

Coexpression of increasing amounts of Cbl resulted in a dose-dependent decrease in tyrosine phosphorylation signal on ZAP-70 (Fig. 4A, first panel, compare lanes 1–4 with lane 14). In contrast, the level of ZAP-70 phosphorylation remained unchanged upon expression of the Cbl TKB domain mutant Cbl-G306E (first panel, compare lanes 5–8 with lane 14). Notably, the ZAP-70-Y292F mutant was completely resistant to the effects of Cbl, as shown by its unchanged level of phosphorylation upon wild-type Cbl coexpression (first panel, compare lanes 9–12 with lane 14). These findings clearly demonstrated that Cbl expression leads to a loss of the phosphorylated pool of ZAP-70. Under these experimental conditions, 25–30% of tyrosine-phosphorylated ZAP-70 was coimmunoprecipitated with Cbl (second panel, lane 4). Notably, the Cbl-70ZJ3 mutant also associated with ZAP-70, in fact at a higher level compared with wild-type Cbl (second panel, compare lanes 1 and 3), and this interaction was also abrogated by the Y292F mutation in ZAP-70 (second panel, lane 2). In each case, immunoprecipitation of HA-tagged Cbl proteins was confirmed by anti-HA immunoblotting (Fig. 3, first panel), and equivalent levels of ZAP-70 expression were confirmed by immunoblotting of whole cell lysate with anti-ZAP-70 Ab (Fig. 3, third panel). Taken together, these results indicated that Cbl associates with ZAP-70 in a TKB-dependent manner, and that the association of both wild-type Cbl as well as the Cbl-70ZJ3 mutant protein requires an intact Tyr292 on ZAP-70.

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FIGURE 4. Negative regulatory effect of Cbl on ZAP-70 tyrosine kinase in COS-7 cells, and the role of Cbl TKB domain and ZAP-70 Tyr292. A, COS-7 cells (4.5 × 10⁵) were cotransfected with pSRαNeo-CD8-ζ (0.7 μg), pdKCR-Lck-505F (0.7 μg), the indicated pAlterMAX-ZAP-70 constructs (0.05 μg), and the indicated amounts of pAlterMAX-HA-Cbl constructs. Lane 15 lacks pdKCR-Lck-505F and lane 16 is a vector-transfected control. Cells were lysed 48 h posttransfection. Thirty-microgram aliquots of whole cell lysates were resolved by SDS-PAGE, and transferred to PVDF membrane. These were immunoblotted with anti-Tyr(P) (first panel), anti-HA (second panel), anti-ZAP-70 (third panel), and anti-ζ (fourth panel) Abs and visualized using enhanced chemiluminescence (ECL).

B and C, The ZAP-70 bands in A for ZAP-70 phosphorylation and for ZAP-70 protein level were quantified by densitometry. The values were expressed as a ratio to the values of the ZAP-70 band in the absence of coexpressed Cbl (A, lane 14), and plotted against the amount (in μg) of transfected Cbl DNA.
conditions, Cbl expression led to a small decrease in CD8-ζ phosphorylation; however, this was seen both in ZAP-70- and ZAP-70-Y292F-transfected cells (first panel, compare lanes 1–4 with lanes 9–12), consistent with the observation that CD8-ζ phosphorylation was Lck dependent.

Anti-ZAP-70 blots of whole cell lysates revealed that the decrease in ZAP-70 tyrosine phosphorylation was paralleled by a decrease in ZAP-70 protein levels (Fig. 4A, third panel, compare lanes 1–4 with lane 14). The decrease in ZAP-70 protein levels was not seen upon coexpression of the Cbl-G306E mutant, nor were protein levels of the ZAP-70-Y292F mutant affected by its coexpression with wild-type Cbl (third panel, compare lanes 5–12 with lane 14). The levels of Cbl protein were lower compared with those of Cbl-G306E protein at equivalent input DNA amounts, while levels of Cbl protein were comparable in cells coexpressing ZAP-70 or the ZAP-70-Y292F mutant (second panel). No major differences in CD8-ζ protein levels were observed when either ZAP-70 or its Y292F mutant was expressed with Cbl or Cbl-G306E (fourth panel). Analysis of cell lysates prepared in SDS-containing lysis buffer revealed that the Cbl-dependent decrease in ZAP-70 protein levels was apparently not due to sequestration in a Triton X-100-insoluble fraction (data not shown).

To quantify the decrease in ZAP-70 phosphorylation and protein levels, densitometric analysis was conducted on the data shown in Fig. 4A. The intensity of ZAP-70 bands (lanes 1–12) was expressed as a ratio to that of the ZAP-70 band in the absence of coexpressed Cbl (lane 14). This analysis further confirmed that coexpression of wild-type Cbl and ZAP-70 results in a Cbl dose-dependent loss of phosphorylated ZAP-70 protein, whereas this effect is not observed when ZAP-70 is coexpressed with Cbl-G306E, nor when the ZAP-70-Y292F mutant is coexpressed with Cbl (Fig. 4, B and C).

Cbl-70Z/3 mutant fails to exert a negative regulatory effect on ZAP-70

The oncogenic 70Z/3 Cbl mutant has been shown to enhance the basal as well as Ca2+-ionophore-induced NF-AT luciferase activity in transfected Jurkat cells (46, 47). It was therefore of interest to ascertain whether or not this Cbl mutant, which prominently associated with ZAP-70 (Fig. 3), retained the ability to negatively regulate ZAP-70. This was tested using the COS cell transfection system described above. In contrast to the effect of overexpressing increasing amounts of wild-type Cbl (Fig. 5A, first panel, compare lanes 1–4 with lane 9), expression of the Cbl-70Z/3 mutant did not result in a decrease in the levels of tyrosine-phosphorylated ZAP-70 (first panel, compare lanes 5–8 with lane 9), even though higher levels of Cbl-70Z/3 protein were expressed compared with wild-type Cbl protein (second panel). In addition, expression of the Cbl-70Z/3 mutant did not result in a decrease in ZAP-70 protein levels (third panel, compare lanes 1–4 with lanes 5–8). Densitometric analysis confirmed the lack of an effect of Cbl-70Z/3 on ZAP-70 phosphorylation and protein levels compared with the effects of wild-type Cbl (Fig. 5, B and C).

Overexpression of Cbl decreases, while Cbl-N increases the anti-CD3-induced NF-AT luciferase activity in Jurkat T cells

Although the analyses in the COS cell system clearly demonstrated a Cbl TKB domain-dependent and ZAP-70-Y292-dependent inhibitory effect of Cbl on ZAP-70 (Fig. 4), we wished to establish this further in the context of the TCR stimulation in a lymphoid cell system. For this purpose, we examined the effects of various Cbl proteins on basal and TCR stimulation-induced activity of the NF-AT luciferase reporter, a ZAP-70-mediated distal readout of TCR signaling (35).

Unstimulated JMC-T cells transiently transfected with increasing amounts of Cbl or Cbl-G306E showed no significant change in the level of NF-AT luciferase reporter activity compared with mock-transfected cells (Fig. 6A). In contrast, a Cbl dose-dependent

FIGURE 5. The effect of Cbl-70Z/3 mutant on ZAP-70 phosphorylation and protein levels in COS-7 cells. A, COS-7 cells were transfected with ZAP-70 and HA-Cbl or HA-70Z/3 expression plasmids, as described in the legend to Fig. 4. Thirty-microgram aliquots of whole cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-Tyr(P) (first panel), anti-HA (second panel), and anti-ZAP-70 (third panel). B and C, The ZAP-70 bands in A for ZAP-70 phosphorylation and for ZAP-70 protein level were quantified by densitometry. The values were expressed as a ratio to the value of the ZAP-70 band in the absence of coexpressed Cbl (A, lane 9), and plotted against the amount (in μg) of transfected Cbl DNA.
FIGURE 6. Effects of Cbl or mutant Cbl expression on TCR-induced NF-AT luciferase activity in transfected JMC-T cells. A, SV-40 T Ag-expressing (JMC-T) Jurkat cells were cotransfected with 10 μg NF-AT luciferase reporter construct together with the indicated amounts of wild-type HA-Cbl (WT) and HA-Cbl-G306E (G306E) in pAlterMAX vector. Cells were stimulated and lysed, and luciferase activity was determined, as described in Materials and Methods. The mean luciferase activity (mean arbitrary light units) of sets of five replicates was determined. These data were expressed as a percentage of the PMA plus ionomycin-induced luciferase activity. The combined SE of both means is displayed. Data are representative of four independent experiments. The expression of each Cbl construct was examined by Western blot analysis. Aliquots of same transfectants that were used in A were cultured for an additional 24 h and then lysed. A total of 50 μg of whole cell lysate was immunoblotted with anti-HA to detect levels of transfected HA-Cbl protein (second panel). B, JMC-T cells were transfected with the indicated amounts of wild-type HA-Cbl-N (WT) and HA-Cbl-N-G306E (G306E), as described above. Data are representative of three independent experiments. The expression of each Cbl-N construct (second panel) was examined by Western blot analysis essentially as described above in A. C, JMC-T cells were cotransfected with 10 μg NF-AT luciferase reporter construct together with 5 μg of HA-Cbl-N, HA-Cbl-N-G306E, HA-Cbl, and HA-Cbl-G306E. Cells were stimulated and luciferase activity measured, as described above. Data are representative of four independent experiments. The expression of each Cbl construct (second panel) was examined by Western blot analysis, as described above in A.
decrease in anti-CD3 Ab-induced NF-AT luciferase activity was observed when wild-type Cbl was expressed in these cells. Notably, the effect of Cbl overexpression was essentially abrogated by the TKB domain-inactivating G306E mutation (Fig. 6A), similar to the effect of this mutation on the negative regulation of ZAP-70 in COS cells (Fig. 4). The lack of effect of the Cbl-G306E mutant was not due to a difference in protein expression (Fig. 6A, second panel).

If Cbl is a relevant negative regulator of ZAP-70 in vivo, overexpression of the Cbl TKB domain alone (Cbl-N) may be expected to enhance signaling by competing with endogenous Cbl for binding to phosphorylated ZAP-70. Indeed, overexpression of Cbl-N, but not its G306E mutant, led to a DNA dose-dependent increase in anti-CD3 Ab-induced NF-AT luciferase activity (Fig. 6B). Neither Cbl-N nor Cbl-N-G306E had an effect on basal NF-AT luciferase activity. These observed differences in the effects of Cbl-N and Cbl-N-G306E were not due to differences in protein levels (Fig. 6B, second panel). A simultaneous comparison of Cbl, Cbl-N, and their G306E mutants further substantiated these results (Fig. 6C).

Cbl-70Z/3 mutant-induced enhancement of basal and anti-CD3-induced NF-AT activity is TKB domain dependent

Previous studies have shown that the oncogenic Cbl mutant 70Z/3 enhances the basal and Ca2+-ionophore-induced NF-AT luciferase activity when overexpressed in Jurkat cells (46); however, its effects on anti-CD3-induced NF-AT luciferase activity and the role of the TKB domain in this process have not been clarified. Because Cbl-70Z/3 clearly and prominently associated with ZAP-70, but not with ZAP-70-Y292F (Fig. 5), it appeared likely that a TKB domain-mediated interaction contributed to the effect of Cbl-70Z/3 on NF-AT activity. As expected (46) (Fig. 5), overexpression of Cbl-70Z/3 in Jurkat cells led to a marked increase in NF-AT luciferase activity in the absence of anti-CD3 triggering (Fig. 7). Anti-CD3 stimulation led to a further increase in the NF-AT luciferase activity; however, the fold increase was markedly lower compared with the vector-transfected control cells, apparently due to high basal activity. Importantly, the G306E mutation in Cbl-70Z/3 led to a complete lack of enhancement of the basal as well as anti-CD3-induced NF-AT luciferase activity when compared with cells transfected with Cbl-70Z/3 (Fig. 7A). Experiments using ZAP-70-deficient p116 T cells reconstituted with ZAP-70 yielded similar results to those obtained using JMC-T cells (Fig. 7B). Therefore, the Cbl-70Z/3 effect on T cell activation, as assessed by NF-AT luciferase activity, appeared to be dependent on the TKB domain. The requirement of the TKB domain for the Cbl-70Z/3-induced increase in NF-AT luciferase activity in the absence of TCR stimulation has been previously observed (47, 48).

A direct role of ZAP-70 in Cbl-dependent decrease of NF-AT luciferase activity in T cells and the requirement of ZAP-70

Because the JMC-T cell line used in the experiments described above expresses ZAP-70 as well as Syk (36), both of which can be targeted by Cbl, the effects of Cbl on NF-AT luciferase could not be conclusively ascribed to ZAP-70. To directly demonstrate that the effects of Cbl-mediated negative regulation reflected its influence on ZAP-70, we utilized the Jurkat E6-1 (Syk negative, ZAP-70 positive)-derived mutant cell line p116, which is ZAP-70 deficient (35). As expected, the lack of ZAP-70 in these cells results in a block of anti-CD3-stimulated increase in NF-AT luciferase activity (Figs. 7B and 8), as well as early activation events such as tyrosine phosphorylation of cellular proteins (data not shown). However, reconstitution of ZAP-70 expression by transient transfection restored the anti-CD3-induced stimulation of NF-AT activity (Figs. 7B and 8). Overexpression of Cbl in the absence of ZAP-70 had no effect on NF-AT luciferase induction. Cotransfection of Cbl and ZAP-70, however, resulted in a decrease in NF-AT luciferase activity compared with that seen with ZAP-70 transfection alone (Fig. 8), confirming results obtained using the JMC-T cell line (Fig. 6). Importantly, coexpression of the Cbl-G306E mutant with ZAP-70 failed to reduce the anti-CD3-stimulated NF-AT luciferase activity, thus confirming the critical role of the Cbl TKB domain.

Previous studies have shown that overexpression of the ZAP-70-Y292F mutant in Jurkat T cells led to a pronounced increase in
both the basal and anti-CD3-stimulated NF-AT luciferase activity when compared with overexpression of wild-type ZAP-70 (31). Indeed, reconstitution of p116 cells with ZAP-70-Y292F mutant led to a comparable phenotype (Fig. 8). Importantly, coexpression of Cbl failed to decrease ZAP-70-Y292F-dependent NF-AT luciferase activity, indicating that this mutant is insensitive to the negative regulatory effects of Cbl. Together, these results show that Cbl can negatively regulate the ZAP-70-dependent activation of T cells, and that this effect is contingent upon an interaction mediated by the Cbl TKB domain binding to the negative regulatory phosphotyrosine 292 in ZAP-70.

Discussion

The Syk/ZAP-70 family of nonreceptor tyrosine kinases plays an essential role in lymphocyte Ag receptor signal transduction and lymphocyte development (44, 49). Therefore, understanding the regulatory mechanisms of these kinases is of considerable interest. Phosphorylation within the kinase domain of ZAP-70 regulates its kinase activity in a positive (Tyr493) as well as a negative (Tyr492) manner (29–31, 33). However, mutation of Tyr292, an in vivo phosphorylation site located between the SH2 and kinase domains, or deletion of this SH2-kinase linker, enhances the level of signaling in vivo without altering the level of kinase activity (29–31, 33, 45). The Cbl protooncoprotein, which has emerged as a negative regulator of tyrosine kinases (1, 2), physically interacts with ZAP-70 (Fig. 3), but did not induce a decrease in ZAP-70 phosphorylation or protein levels (Fig. 5). Similarly, Cbl-N interacts with ZAP-70 and Syk, but does not lead to a reduction in the autophosphorylated pool of ZAP-70 (31). These findings led us to hypothesize that Cbl recruitment represents an activation-induced mechanism to negatively regulate ZAP-70. In this study, we provide direct experimental evidence for this hypothesis.

The negative regulatory effect of Cbl on ZAP-70 was demonstrated in two different cellular systems using distinct readouts. First, we used HA-Cbl-overexpressing Jurkat T cell clones to study the effect of Cbl on early events of T cell activation. Each of the three Cbl-overexpressing clones showed a reduction in the level of anti-CD3-induced tyrosine phosphorylation of cellular substrates compared with the parental T cell line (Fig. 2). Specifically, we observed a reduction in the level of ZAP-70 tyrosine phosphorylation in all three Cbl-overexpressing clones. The inhibitory effect of Cbl overexpression was also observed using a distal readout of T cell activation, the phosphorylation of MAP kinases.

These results in Jurkat T cell transfectants, and previous findings that the Cbl TKB domain interacts with the negative regulatory linker phosphorylation site Y292 in ZAP-70 (13) were consistent with a role for Cbl as a negative regulator of ZAP-70. Direct evidence for this idea was provided by analyses in the COS cell reconstitution system, in which coexpression with Cbl led to a marked reduction in the autophosphorylated pool of ZAP-70 (Fig. 4). Another measure of Cbl-dependent negative regulation in COS cells was provided by a decrease in ZAP-70 protein levels, analogous to Cbl-dependent decrease in Syk protein levels in the COS cell system (14). Although the mechanisms of reduction in Syk/ZAP-70 protein levels in the COS cell system are presently unclear, it provides a useful index of the effect of Cbl on these tyrosine kinases in COS cells.

The Cbl-dependent negative regulation of ZAP-70 in the COS cell system required the conserved TKB domain of Cbl and phosphorylated Tyr292 in ZAP-70, both of which are required for the physical association of these two proteins (Fig. 4). However, the TKB domain-mediated physical association of Cbl with ZAP-70 is not by itself sufficient for negative regulation, as demonstrated by analyses of the oncogenic Cbl-70Z/3 mutant. Cbl-70Z/3 carries a 17-aa deletion near the N-terminal boundary of the highly conserved RING finger domain. This mutant prominently associated with ZAP-70 (Fig. 3), but did not induce a decrease in ZAP-70 phosphorylation or protein levels (Fig. 5). Similarly, Cbl-N interacts with ZAP-70 and Syk, but does not lead to a reduction in the autophosphorylated pool of these tyrosine kinases (S. Ota, N. Rao, and H. Band, unpublished results). Thus, domains C terminal to the TKB domain, such as the RING finger and/or other regions, appear to be important for the negative regulatory effect of Cbl on ZAP-70. Recent analyses have revealed that the TKB and RING finger domains are sufficient for Cbl-dependent negative regulation of Syk in a COS cell system (S. Ota, N. Rao, and H. Band, unpublished results).

To directly assess the negative regulatory effect of Cbl on ZAP-70 function in T cells, further analyses were conducted in a Jurkat T cell line and its ZAP-70-deficient mutant. Using the TCR-induced NF-AT luciferase reporter activity as a measure of ZAP-70-dependent T cell activation, we demonstrated that wild-type Cbl expression led to a dose-dependent inhibition of T cell activation in a TKB domain-dependent manner (Fig. 6, A and C). The importance of the TKB domain-mediated interaction of Cbl with ZAP-70 was also revealed by the inability of the 70Z/3 Cbl mutant with an inactive TKB domain (G306E mutation) to enhance basal and TCR-induced NF-AT luciferase activity (Fig. 7). Other groups have also recently shown that the G306E mutant of Cbl-70Z/3 was unable to enhance the basal NF-AT luciferase activity in Jurkat cells (47, 48); however, the effect of Cbl-70Z/3 on TCR stimulation-induced NF-AT activation was not investigated in these studies (46, 47). Finally, the expression of the Cbl TKB domain (Cbl-N) by itself led to a substantial increase in TCR-induced
NF-AT luciferase activity, and this effect was abrogated by the G306E mutation (Fig. 5, B and C). Apparently, the mutant Cbl proteins, such as Cbl-70Z1 and Cbl-N, bind to ZAP-70 and prevent the endogenous wild-type Cbl from exerting a negative regulatory effect. This phenotype of oncogenic Cbl mutants is strongly indicative of a negative regulatory role of the endogenous Cbl for ZAP-70.

Given the presence of endogenous ZAP-70 in Jurkat JMC-T cells, and the expression of Syk in the particular cell line used in our analyses (36 and data not shown), we extended our analyses to a ZAP-70 and Syk-deficient Jurkat T cell line, p116 (35). As previously reported (35), stimulation of this cell line through the TCR did not induce NF-AT luciferase activity, and Cbl expression by itself had no effect (Fig. 8). The expression of wild-type ZAP-70 reconstituted the TCR-induced NF-AT luciferase activity; Cbl coexpression, but not Cbl-G306E coexpression, reduced the NF-AT activity. As expected from studies in wild-type Jurkat cells (31), the ZAP-70 Y292F mutant demonstrated a hyperresponsive phenotype when expressed in p116 cells (Fig. 8). Notably, coexpression of Cbl failed to down-regulate the ZAP-70-Y292F mutant-mediated NF-AT luciferase activation in p116 cells. The reconstitution studies in p116 cells demonstrate that Cbl indeed impinges on ZAP-70 function via a mechanism that requires an intact Cbl TKB domain and an intact negative regulatory phosphorylation site, Tyr292, in ZAP-70.

Our conclusion that Cbl is a negative regulator of ZAP-70 is consistent with the phenotype of c-cbl-deficient mice, which display hypercellularity in thymus and spleen (22). The thymocytes of these mice displayed higher levels of tyrosine-phosphorylated proteins, including ZAP-70, upon TCR stimulation. Furthermore, when the c-cbl deficiency was bred into TCR transgenic mice, an enhancement of positive selection of CD4+ thymocytes was observed (23). Our studies, however, provide direct evidence that the negative regulatory effect of Cbl on ZAP-70 is indeed exerted via its linker phosphorylation site.

Demonstration of a role for Cbl as a negative regulator of ZAP-70 is of considerable interest in relation to the shared and divergent regulation of Syk and ZAP-70 (49). Compared with ZAP-70, Syk has a substantially higher intrinsic kinase activity and is less dependent on Src family kinases for its activation (49–52). However, Cbl does function as a negative regulator of Syk, as demonstrated by experiments in a transfected RBL 2H3 mast cell line, COS cells, and more recently in lymphoid cells (14, 28) (S. Ota and H. Band, unpublished results). Notably, Cbl association with Syk also requires an intact Cbl TKB domain and Syk Tyr323; the latter corresponds to ZAP-70 Tyr292 (14, 15, 32, 49). Thus, Cbl represents a negative regulator that is recruited to activated ZAP-70/Syk kinases via the regulatory phosphorylation sites outside their kinase domains.

Coexpression of Cbl and ZAP-70 in COS cells resulted in a decrease in the amount of ZAP-70 (49), similar to our earlier observation of Cbl-dependent decreases in the levels of coexpressed Syk (14). In both cases, the Cbl TKB domain and the cognate binding sites on Syk/ZAP-70 are required for a decrease in the protein levels of these tyrosine kinases. However, we did not observe a detectable decrease in ZAP-70 protein levels in Cbl-overexpressing Jurkat T cells, even though a clear reduction in ZAP-70 phosphorylation was observed. This discrepancy might reflect the activation of a smaller pool of ZAP-70 in T cells, as opposed to COS cells, in which the coexpression of Lck probably leads to activation of a larger pool of ZAP-70. Consistent with this idea, a small decrease in Syk protein levels was observed in anti-IgM-stimulated Ramos B cells, and this decrease was accentuated when Cbl was overexpressed (S. Ota and H. Band, unpublished results). Furthermore, a recent report showed a decrease in the levels of ZAP-70 in Ag-activated T cells, although the role of Cbl in these effects was not investigated (53). It is, however, possible that the Cbl-induced loss of ZAP-70 protein that we observed is not mechanistically relevant to T cells. Further analyses will be required to address these possibilities.

In conclusion, our studies show that the negative regulatory phosphorylation site within ZAP-70 provides a means to recruit Cbl as a negative regulator in activated T lymphocytes, and that loss of this site renders ZAP-70 insensitive to Cbl-mediated negative regulation. Further studies of the Cbl-ZAP-70 interaction are therefore likely to elucidate a physiologically relevant pathway of regulating tyrosine kinase function in T cells.

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