

# Negative regulation of Lck by Cbl ubiquitin ligase

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**The Cbl-family ubiquitin ligases function as negative regulators of activated receptor tyrosine kinases by facilitating their ubiquitination and subsequent targeting to lysosomes. Cbl associates with the lymphoid-restricted nonreceptor tyrosine kinase Lck, but the functional relevance of this interaction remains unknown. Here, we demonstrate that T cell receptor and CD4 coligation on human T cells results in enhanced association between Cbl and Lck, together with Lck ubiquitination and degradation. A Cbl<sup>-/-</sup> T cell line showed a marked deficiency in Lck ubiquitination and increased levels of kinase-active Lck. Coexpression in 293T cells demonstrated that Lck kinase activity and Cbl ubiquitin ligase activity were essential for Lck ubiquitination and negative regulation of Lck-dependent serum response element-luciferase reporter activity. The Lck SH3 domain was pivotal for Cbl-Lck association and Cbl-mediated Lck degradation, with a smaller role for interactions mediated by the Cbl tyrosine kinase-binding domain. Finally, analysis of a ZAP-70-deficient T cell line revealed that Cbl inhibited Lck-dependent mitogen-activated protein kinase activation, and an intact Cbl RING finger domain was required for this functional effect. Our results demonstrate a direct, ubiquitination-dependent, negative regulatory role of Cbl for Lck in T cells, independent of Cbl-mediated regulation of ZAP-70.**

**P**rotein tyrosine kinase (PTK) activation is an early and necessary event for cellular activation upon engagement of antigen receptors such as the B and T cell receptors (TCR) and Fc receptors (1). The initial event involves the activation of membrane-anchored Src-family kinases (SFKs), such as Lck and Fyn, which phosphorylate the immunoreceptor tyrosine-based activation motifs within the signaling subunits of the receptor, thus creating docking sites to recruit Syk or ZAP-70 PTKs. Genetic and biochemical analyses have established that serial SFK and Syk/ZAP-70 activation is required for antigen receptor signaling (1).

Lck plays a particularly important role in the immune system, and this lymphoid-restricted SFK plays a vital role in T cell development and function (2). Although accentuation of the CD4<sup>-</sup>CD8<sup>-</sup> T cell developmental block in Lck<sup>-/-</sup> mice by concurrent Fyn-deficiency suggests partial redundancy, Fyn does not restore peripheral T cell activation in Lck<sup>-/-</sup> mice, thus indicating an essential, nonredundant role of Lck in T cell activation (3–5).

Lck activation by *Herpes saimari* tyrosine kinase-interacting protein (TIP)-transforming protein and mutational analysis of Lck and other SFKs has established that their unregulated activity results in oncogenicity (6, 7). Thus, precise regulation of Lck is vital for physiological function. Intramolecular SH2 domain-binding to the negative regulatory phosphotyrosine residue near the C terminus, and the SH3 domain-binding to the SH2-kinase linker region maintains SFKs in an inactive, closed conformation, accounting for their basal repressed state. On cellular activation, these intramolecular interactions cease resulting in derepression of the kinase domain while concurrently promoting SH2 and SH3 domain-mediated protein–protein interactions that are essential for signal transmission (8).

In contrast to mechanisms of basal repression and activation that are well supported by crystal structural studies (8), mechanisms of SFK inactivation have been less clear. Tyrosine phosphatases, such

as SHP-1, provide one likely mechanism (9); however, it is unclear whether dephosphorylation is sufficient to revert activated Lck back into its inactive state, a process that would also require C-terminal Src kinase (CSK)-mediated phosphorylation of Lck and possibly cellular chaperones such as Hsp90 (10). Recent studies indicate that the Cbl protein family provides a new mode of negatively regulating the activated pools of SFKs (11, 12).

With three distinct mammalian members, the Cbl family of multidomain-signaling proteins is highly conserved in sequence and domain architecture from *Caenorhabditis elegans* to man (13, 14). The conserved N-terminal tyrosine kinase-binding (TKB) domain binds to activation-induced phosphotyrosine motifs and the linker helix and a RING finger domain mediate physical interaction with the E2 ubiquitin (Ub) conjugating enzymes of the Ub pathway (15). Thus Cbl can function as an E3 Ub ligase toward activated PTKs bound to the Cbl TKB domain (15). Cbl-mediated ubiquitination of activated receptor tyrosine kinases serves as a lysosomal targeting signal (16), whereas ubiquitination of nonreceptor PTKs Syk and ZAP-70 targets them for proteasomal degradation (17, 18).

Several SFKs, such as Fyn, Src, Lck, and Lyn, interact with Cbl by way of the SFK SH3 domain binding to the Cbl proline-rich region, and possibly by way of the SFK SH2 domain binding to phosphorylated Cbl (13). Recent results have shown that Cbl can dramatically reduce the pool of active Fyn through enhanced degradation (11), suggesting a role for Cbl in negatively regulating SFKs. However, analyses of Src-dependent cell spreading and migration in macrophages (19) and bone resorption in osteoclasts (20) have suggested a positive role of Cbl in these responses downstream of Src. Therefore, it is unclear whether negative regulation of Fyn by Cbl is a specialized case or generalizable to other SFKs such as Lck. This question is of obvious importance because the vast majority of cellular Lck is anchored to the plasma membrane, whereas Fyn and Src localize primarily to intracellular vesicles (21). Furthermore, only Lck directly associates with T cell coreceptors CD4/CD8. Here, we demonstrate that Cbl and Lck associate upon TCR/CD4 activation and Lck is ubiquitinated and degraded by the proteasome. These studies support a novel role for Cbl-dependent ubiquitination and degradation in the negative regulation of Lck. Together with previous results, using Fyn, this study suggests a general role for Cbl to regulate SFKs.

## Materials and Methods

**Cells.** The 293T human embryonic kidney epithelial cells, T cell lines 230 and 206 from Cbl<sup>+/+</sup> and Cbl<sup>-/-</sup> mice, the human CD4<sup>+</sup> T cell clone SPF1, and the ZAP-70-deficient Jurkat T cell

Abbreviations: IP, immunoprecipitation; PTK, protein tyrosine kinase; SFK, Src-family kinase; SRE, serum response element; TCR, T cell receptor; TKB, tyrosine kinase binding; Ub, ubiquitin; WT, wild type; HA, hemagglutinin; MAPK, mitogen-activated protein kinase.

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line expressing the SV40 T antigen (p116-T) were all maintained as described (11, 18, 22).

**Antibodies.** The antibodies used were: monoclonal antibody (mAb) 12CA5 [anti-influenza hemagglutinin (HA)]; mAb 4G10 (anti-pTyr); mAb SPV-T3b (anti-CD3 $\epsilon$ ); mAb OKT4 (anti-CD4); mAb W6/32 (anti-MHC I); mAb anti-Ub from Covance; rabbit polyclonal antibody anti-p44/42 mitogen-activated protein kinase (MAPK), polyclonal antibody anti-p44/42 phospho-MAPK, and polyclonal antibody anti-phospho-Src from Cell Signaling Technology (Beverly, MA) and mAb anti-Lck and polyclonal antibody anti-Cbl from Santa Cruz Biotechnology.

**Expression Plasmids.** The Cbl expression constructs in pAlterMAX and pCDNA3 vector backbone have been described (11, 17, 23). To generate pAlterMAX-Lck constructs, pDKCR Lck and mutant constructs (18) were used as templates for PCR followed by cloning into the pAlterMAX vector. The Lck SH2 (R154K), SH3 (W97A), and double mutant were generated by using Quickchange Mutagenesis (Invitrogen). The plasmid encoding HA-Ub was kindly provided by D. Bohmann (European Molecular Biology Organization, Heidelberg, Germany).

**Cell Lysis.** Cell lysates were prepared in one of the following buffers as indicated in the figure legends: Triton lysis buffer (11); RIPA buffer (0.15 M NaCl/0.05 M Tris, pH 7.5/1% Triton X-100/1% sodium deoxycholate/0.1% SDS), Triton lysis buffer containing 0.1% SDS and 0.5% deoxycholate; and SDS lysis buffer, Triton lysis buffer containing 1% SDS.

**Transient Expression.** The 293T cells were transfected by using the calcium phosphate method, and p116T cells were transfected by electroporation (11). Cell lysates were prepared 48 h posttransfection.

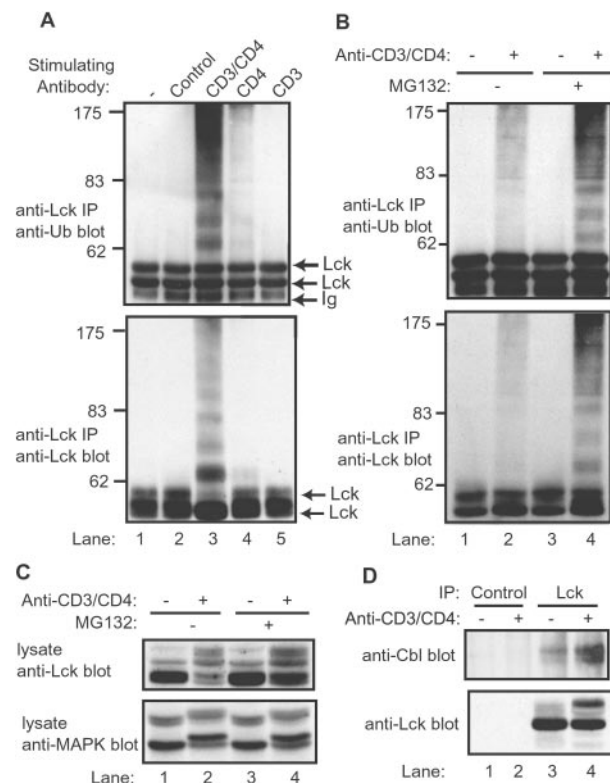
**T Cell Stimulation.** The p116-T cells were stimulated through the TCR by adding SPV-T3b antibody for the indicated times, and cells were lysed. SPF1 T cells were washed in RPMI medium 1640 containing Hepes and glutamine, resuspended at  $50 \times 10^6$ /ml, and incubated with the following mAb (ascites 1:100) SPV-T3b, OKT4, or W6/32 (anti-MHC I) control. After incubation on ice for 30 min, the cells were washed, warmed to 37°C, and bound antibodies were cross-linked by using rabbit anti-mouse antibody.

**Immunoprecipitation, Gel Electrophoresis, and Immunoblotting.** Immunoprecipitations (IPs) were performed as described (17). The immunoprecipitated proteins and total cell lysates were resolved by SDS/PAGE, transferred to poly(vinylidene difluoride) membranes (NEN), immunoblotted with the indicated antibodies and visualized as described (17). Band intensity was quantified by densitometry by using SCIONIMAGE3B (www.Scioncorp.com).

**Luciferase Assay.** The 293T cells were transfected with a serum response element (SRE)-luciferase reporter construct and the appropriate Cbl and Lck constructs by using the calcium phosphate method, and assays were performed as described (11).

## Results

**Lck Ubiquitination and Association on TCR Stimulation.** We asked whether coligation of the TCR and CD4 on a normal CD4<sup>+</sup> human T cell clone, SPF1, induced Lck ubiquitination. Although Lck ubiquitination was undetectable on CD3 ligation, similar to controls, CD4 ligation resulted in a low level of Lck ubiquitination seen as distinct higher-molecular-weight species together with a smear (Fig. 1A Upper). Notably, CD3/CD4 coligation resulted in easily detectable Lck ubiquitination. Anti-Lck immunoblotting revealed the higher-molecular-weight bands and

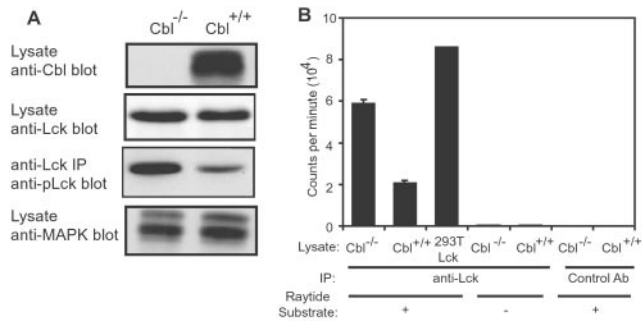


**Fig. 1.** Lck associates with Cbl and is ubiquitinated upon TCR stimulation in SPF1 T cells. (A) Resting CD4<sup>+</sup> human SPF1 T cells that had been incubated for 12 h in the absence of IL-2 were stimulated by cross-linking with no antibody (–), control antibody, or anti-CD3/4 antibodies for 10 min at 37°C and then lysed in SDS lysis buffer. Anti-Lck IPs from 1-mg aliquots of lysates were serially probed with an anti-Ub (Top) and anti-Lck antibody (Bottom). (B) SPF1 T cells were treated with 50  $\mu$ M MG132 (+) or dimethyl sulfoxide vehicle control (–) for 3 h before stimulation for 10 min. IPs were performed and immunoblotted as above. (C) Equal amounts of protein lysates from B were serially probed with anti-Lck and anti-MAPK antibodies. (D) SPF1 T cells were stimulated for 5 min., lysed in Triton lysis buffer, and anti-Lck and isotype matched control IPs were immunoblotted with anti-Cbl (Top) or anti-Lck antibody (Bottom).

smear to be Lck (Fig. 1A Lower, lane 3). The more intense Ub vs. Lck signal on higher-molecular-weight species represents an increased Ub epitope density on multiubiquitinated Lck. Anti-CD3/CD4-induced Lck ubiquitination was also observed by using human lymphoblast preparations freshly derived from peripheral blood (data not shown). The induction of Lck ubiquitination by various stimuli corresponded to their ability to induce early tyrosine phosphorylation events (data not shown).

Pretreatment of SPF1 T cells with the proteasome inhibitor MG132 resulted in a marked enhancement of Lck ubiquitination compared with control (Fig. 1B Upper). Concomitantly, MG132 treatment resulted in enhanced detection of the higher-molecular-weight species in an anti-Lck blot (Fig. 1B Lower). Anti-Lck immunoblotting of whole cell lysates also revealed that signals corresponding to unmodified Lck species decreased on CD3/4 coligation, apparently reflecting the shift into higher-molecular-weight bands and protein degradation (Fig. 1C, lane 2); notably, MG132 treatment led to a slight but reproducible increase in the intensity of this band (Fig. 1C, compare lanes 2 and 4). MAPK levels were comparable in the presence and absence of MG132, indicating that changes in Lck protein were specific. Altogether, these findings demonstrate that TCR plus CD4 coligation induces Lck ubiquitination in normal T cells, and that ubiquitination targets Lck to the proteasome.

The Lck SH3 domain binds to Cbl *in vitro*, and the two proteins



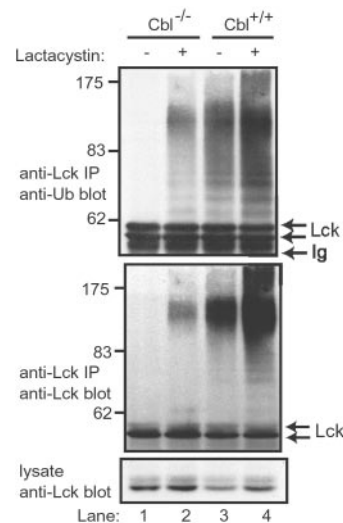
**Fig. 2.** Cbl<sup>-/-</sup> T cells have increased levels of kinase-active Lck. (A) Cbl<sup>-/-</sup> and Cbl<sup>+/+</sup> T cells were lysed in RIPA buffer and equal amounts (50 μg) of protein lysates were immunoblotted with anti-Cbl (top panel), anti-Lck (second panel) and anti-MAPK (bottom panel) antibodies. Anti-Lck IPs from 250 μg of protein lysate was immunoblotted with anti-phospho-Lck (third panel). (B) Anti-Lck or isotype matched control IPs from lysates used in A, or a positive control lysate from transiently transfected 293T cells, were subjected to *in vitro* kinase assays and the incorporation of <sup>32</sup>P signal of [<sup>32</sup>P]ATP into a synthetic Raytide substrate (+) or negative control substrate (-) was quantified. Results are expressed as the mean ± 1 SD of three replicates.

associate *in vivo* (24), suggesting the possibility that Cbl Ub ligase may negatively regulate Lck by means of ubiquitination. In unstimulated SPF1 cells, a low but detectable level of Cbl was coimmunoprecipitated with Lck (Fig. 1D, lane 3). Notably, this association substantially increased upon anti-CD3/CD4 stimulation (Fig. 1D, compare lanes 3 and 4). Lack of Cbl coimmunoprecipitation in control IPs indicated that the Cbl-Lck association was specific.

**Increased Levels of Activated Lck in Cbl<sup>-/-</sup> T Cells.** Given the TCR/CD4-induced Lck ubiquitination and Cbl-Lck association, we asked whether Cbl is required for Lck ubiquitination and degradation by comparing Lck levels in thymocyte-derived, immortalized Cbl<sup>-/-</sup> and Cbl<sup>+/+</sup> T cell lines (11). Anti-Cbl immunoblotting of lysates confirmed the expected Cbl deficiency in the Cbl<sup>-/-</sup> cell line, whereas anti-MAPK immunoblotting showed equivalent sample loading (Fig. 2A). Anti-Lck immunoblotting revealed a modestly higher level of total Lck protein in Cbl<sup>-/-</sup> T cells compared with Cbl<sup>+/+</sup> cells (Fig. 2A, second panel). However, immunoblotting of anti-Lck IPs with an antibody against the phosphorylated activation loop (thus reactive only with activated Lck) revealed a markedly higher level of active Lck in Cbl<sup>-/-</sup> compared with Cbl<sup>+/+</sup> cells (Fig. 2A, third panel).

To assess directly whether the increased level of autophosphorylated Lck in Cbl<sup>-/-</sup> cells represented accumulation of kinase-active Lck, anti-Lck IPs were performed with cell lysates (same as Fig. 2A) prepared in SDS-containing lysis buffer (to disrupt protein complexes), and subjected to *in vitro* kinase assays. Negligible [<sup>32</sup>P]ATP incorporation was seen with negative control IPs or if substrate peptide was omitted (Fig. 2B). Notably, anti-Lck IPs from Cbl<sup>-/-</sup> T cell lysates showed 3-fold higher kinase activity than those from Cbl<sup>+/+</sup> T cells. The accumulation of kinase-active Lck as a result of Cbl deficiency supported a role for Cbl in the ubiquitination and degradation of activated Lck.

Next we asked whether accumulation of Lck in the Cbl<sup>-/-</sup> T cell line was related to inefficient ubiquitination. A low but detectable Lck Ub signal was observed in Cbl<sup>+/+</sup> T cells in the absence of the proteasome inhibitor lactacystin; this signal increased markedly on lactacystin treatment (Fig. 3 Top, compare lanes 3 and 4). In contrast, the Lck Ub signal was essentially undetectable in Cbl<sup>-/-</sup> T cells, and the signal remained very low even after lactacystin treatment (Fig. 3 Top, compare lanes 1 and



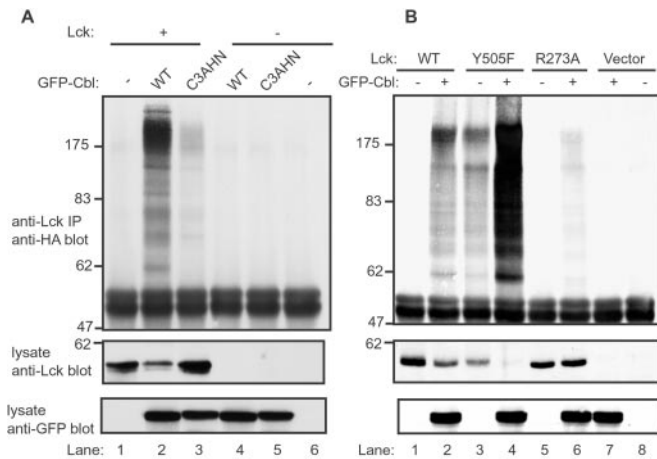
**Fig. 3.** Impaired Lck ubiquitination in Cbl<sup>-/-</sup> T cells. Cbl<sup>-/-</sup> and Cbl<sup>+/+</sup> T cells were incubated with 10 μM Lactacystin (+) or dimethyl sulfoxide control (-) as well as 0.1 mM orthovanadate for 5 h and then lysed in RIPA buffer. Anti-Lck IPs from 1 mg aliquots of lysate were immunoblotted with anti-Ub antibody (Top), followed by anti-Lck antibody (Middle). Equal aliquots (30 μg) of cell lysates were immunoblotted with anti-Lck antibody (Bottom).

2). The accumulated Lck-Ub could also be visualized with an anti-Lck immunoblot (Middle). Furthermore, anti-Lck immunoblotting of whole cell lysate from Cbl<sup>+/+</sup> cells indicated an accumulation of Lck protein upon lactacystin treatment compared with no change in Cbl<sup>-/-</sup> cells (Bottom). These findings strongly support the conclusion that Lck ubiquitination and protein levels in T cells is controlled by the presence of Cbl protein.

**Ubiquitination of Lck in a Reconstitution System.** The results in T cells strongly suggested that Lck ubiquitination is a result of its interaction with Cbl. To address this suggestion directly, we compared the ability of the wild-type (WT) Cbl protein vs. the Ub ligase-deficient RING finger mutant C3AHN (23) to target Lck for ubiquitination in transfected 293T cells (Fig. 4A). Although relatively little Ub signal was detected on Lck when it was cotransfected with vector (control), coexpression with WT Cbl led to strong Ub signal on Lck, accompanied by a reduction in the level of Lck protein (Fig. 4A, compare lanes 1 and 2). In contrast, the Cbl C3AHN mutant was unable to induce Lck ubiquitination or a decrease in Lck protein level (compare lane 2 with lane 3), despite expression at levels comparable with that of WT Cbl (Bottom).

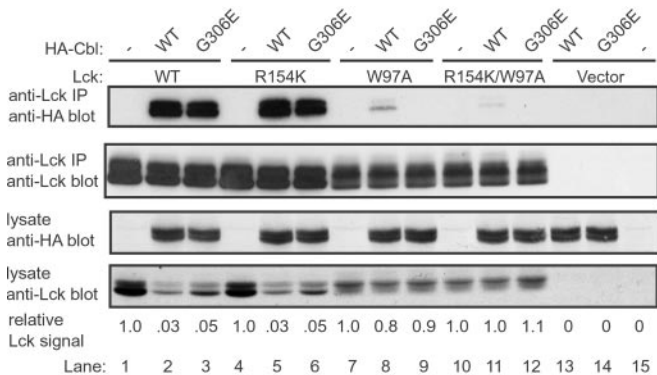
Next, we directly tested the role of Lck kinase activity in ubiquitination assays by comparing WT Lck with its kinase active (Y505F) and kinase dead (R273A) mutants (Fig. 4B). WT Lck was ubiquitinated and degraded when Cbl was coexpressed, as we had already found. In contrast, constitutively active Lck (Y505F) showed detectable ubiquitination even in the absence of cotransfected Cbl, and this ubiquitination was markedly enhanced when Cbl was coexpressed (compare lane 3 with lane 4). Kinase dead Lck (R273A) was essentially insensitive to Cbl-mediated ubiquitination or degradation (compare lane 6 with lanes 2 and 4). Together, these findings demonstrate that Cbl-mediated ubiquitination of Lck depends on Lck kinase activity and an intact Cbl RING finger domain.

Because Cbl can associate with SFKs through multiple interactions (14), we wanted to determine the relative importance of each interaction for Cbl-mediated degradation of Lck. Coexpression of WT Lck, SH2 mutant (R154K), SH3 mutant

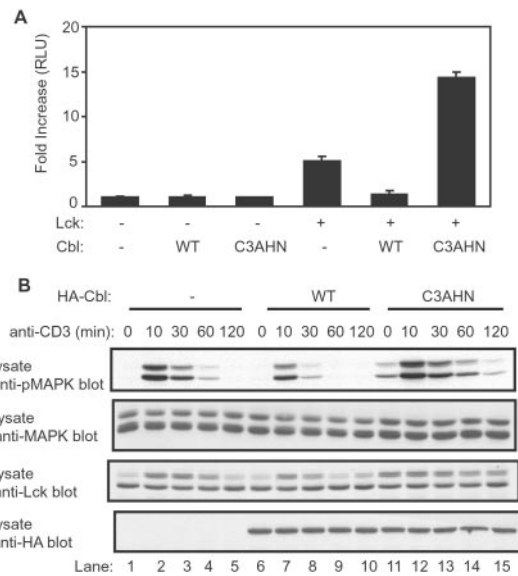


**Fig. 4.** Cbl-dependent ubiquitination of Lck in 293T cells is dependent on the Cbl RING finger domain and Lck kinase activity. (A) 293T cells were transfected with plasmids encoding HA-Ub (5  $\mu$ g), Lck (0.2  $\mu$ g), and 3  $\mu$ g of GFP-Cbl (WT), GFP-Cbl-C3AHN RING finger mutant, or a GFP control (-). Cells were lysed in RIPA buffer, and anti-Lck IPs from 800- $\mu$ g aliquots of lysate protein were immunoblotted with anti-HA antibody (Top). Equal aliquots (30  $\mu$ g) of cell lysates were immunoblotted with anti-Lck antibody (Middle) followed by anti-GFP antibody (Bottom). Control GFP is not included in the blot. (B) 293T cells were transfected with plasmids encoding HA-Ub (5  $\mu$ g), Lck (WT), kinase active (Y505F), and kinase dead (R273A) (0.2  $\mu$ g each), and GFP-Cbl or a GFP control (-) (3  $\mu$ g). Cells were lysed in RIPA buffer, and immunoblots of anti-Lck IPs were performed as in A.

(W97A), or SH3/SH2 double mutant with WT Cbl or the Cbl TKB domain mutant (G306E) followed by coimmunoprecipitation revealed that disruption of the Lck SH3 domain severely abrogated association with Cbl (Fig. 5, compare lane 8 with lane 2). Moreover, disruption of both the SH2 and SH3 domain nearly completely abrogated Lck association with Cbl (compare lane 12 with lane 8). Compared with the SH3 domain mutant, a mutated Lck SH2 domain alone did not significantly disrupt Cbl-Lck association, whereas disruption of the Cbl TKB domain in all



**Fig. 5.** Relative contribution of Lck SH2 and SH3 domains and the Cbl TKB domain toward Cbl-mediated Lck degradation. 293T cells were transfected with plasmids encoding HA-Cbl (1  $\mu$ g) and Lck (WT), SH2 (R154K), SH3 (W97A), or double mutants (R154K/W97A) (0.5  $\mu$ g each). Cells were lysed in Triton lysis buffer and anti-Lck IPs from 1-mg aliquots of lysate protein were immunoblotted with anti-HA antibody (Top) and anti-Lck antibody (Upper Middle). Aliquots (20  $\mu$ g) of lysate protein were immunoblotted with anti-HA antibody (Lower Middle). For degradation, 293T cells were transfected as above, but with 0.2  $\mu$ g of the indicated Lck plasmids and lysed with RIPA buffer. 20  $\mu$ g aliquots of lysate protein were immunoblotted with anti-Lck antibody (Bottom). Lck protein levels were quantified by densitometry and are expressed relative to each Lck protein in the absence of coexpressed Cbl.



**Fig. 6.** The RING finger domain is required for Cbl-dependent negative regulation of Lck. (A) 293T cells were transfected with plasmids encoding the SRE-luciferase reporter (5  $\mu$ g) and the indicated combinations of Lck (0.15  $\mu$ g), HA-Cbl, HA-Cbl-C3AHN and HA-Cbl-70Z (1  $\mu$ g) or pAlterMAX vector (-). Luciferase activity was expressed relative to activity of lysates transfected with the reporter in the absence of Lck or Cbl. Results represent the mean  $\pm$  one SD of five replicate transfections. (B) Jurkat-derived ZAP-70-deficient p16-T cells, were transfected with 15  $\mu$ g of plasmid DNA encoding HA-Cbl, HA-Cbl-C3AHN, or pAlterMAX vector (-). Cells were either left unstimulated or stimulated for the indicated times with anti-CD3 antibody before lysis in RIPA buffer. Equal aliquots of cell lysates (25  $\mu$ g) were subjected to anti-phospho-MAPK (Top), anti-MAPK (Upper Middle), anti-Lck (Lower Middle), and anti-HA (Bottom) immunoblotting.

cases slightly decreased Cbl-Lck association. The association data correlated with the ability of Cbl or its TKB domain mutant (G306E) to mediate Lck degradation, as assessed by quantification of Lck protein levels by densitometry (Fig. 5 Bottom). Both WT Lck and the SH2 domain mutant were equally sensitive to Cbl-mediated degradation (Fig. 5 Bottom, compare lanes 2 and 5). The Lck SH3 mutant was markedly resistant to Cbl-mediated degradation, whereas mutation of both the SH2 and SH3 domains completely blocked degradation (compare lanes 8 and 11). Moreover, the mutation of the Cbl TKB domain slightly blocked degradation of Lck. Together with findings on other SFKs (11, 20), these data suggest that the Lck SH3 domain is the primary mediator of association between Cbl and Lck. The Cbl TKB domain, which specifically binds phosphopeptide motifs (hence phosphorylated Lck), is also involved in this association and subsequent degradation, whereas the Lck SH2 domain plays a much smaller role in Cbl-Lck association and degradation.

**The Cbl RING Finger Domain-Dependent Negative Regulation of Lck Function in 293T and Jurkat T Cells.** To investigate the functional implications of Cbl-mediated ubiquitination of Lck function, we first compared the effects of WT Cbl and its RING finger domain mutant on Lck kinase-dependent transactivation of the SRE-luciferase reporter (25). Ectopic expression of Lck protein in 293T cells led to a nearly 5-fold increase in SRE-luciferase activity compared with mock-transfected cells (Fig. 6A). The Lck-induced increase in SRE-luciferase activity was suppressed to near basal levels upon coexpression of WT Cbl. In contrast, coexpression of the Cbl C3AHN RING finger mutant failed to reduce the Lck-dependent SRE-luciferase reporter activity and, instead, substantially enhanced it (Fig. 6A).

Next, we examined the ability of Cbl to regulate Lck function

in a TCR-driven signaling pathway. Studies have established that MAPK activation on TCR cross-linking in Jurkat T cells is Lck-mediated but independent of ZAP-70 (26, 27). Therefore, we used the ZAP-70-deficient Jurkat-derived T cell line, p116, to assess the effect of Cbl on Lck-mediated cellular activation in T cells. This system also avoids any potential effects caused by Cbl-mediated negative regulation of ZAP-70 (18), a downstream target of Lck. As expected (26, 27), MAPK activation was specifically induced by anti-CD3 cross-linking (Fig. 6B). Cbl overexpression led to a decrease in the intensity of peak phospho-MAPK signal with a markedly rapid loss of signal over time (30 min vs. 60 min in control). In contrast, although cells transfected with the C3AHN mutant exhibited only slightly enhanced peak phospho-MAPK signal, the signals remained elevated for a considerably longer time (120 min vs. 60 min in control). Furthermore, these results correlated with a decrease of phosphorylated, active Lck (*Upper*) in the presence of Cbl, and enhanced levels of active Lck in the presence of the Cbl RING finger mutant (Fig. 6B *Upper Middle*). Overall, the data in 293T and Jurkat T cell systems demonstrate that Cbl functions as a negative regulator of Lck by its RING finger domain-mediated Ub ligase activity.

## Discussion

Lck, a lymphoid-restricted SFK, is essential for T cell development and is indispensable for mature T cell activation (1). The mechanisms that control Lck function are therefore central to regulation of the immune response. The studies presented here demonstrate that Cbl functions as a negative regulator of Lck. We also demonstrate that negative regulation of Lck by Cbl involves ubiquitination and proteasome-mediated degradation of the active pool of Lck and depends on the Ub ligase activity of Cbl. Thus, our studies identify the most proximal T cell PTK as a direct target of negative regulation by Cbl, the prototype of the Cbl family of negative regulators. Recent transfection analyses indicate that Fyn and Src, two ubiquitously expressed SFKs, are also targets of Cbl-induced ubiquitin-dependent degradation (11, 12), raising a strong likelihood that Cbl functions as a general negative regulator of SFKs. Cbl-mediated negative regulation of Lck and other SFKs is likely to provide a regulatory role complementary to other mechanisms, such as CSK-dependent repression and various tyrosine phosphatases.

In contrast to Cbl-mediated ubiquitination of receptor tyrosine kinases, which involves addition of only a few Ub moieties and serves as a sorting signal for transport to lysosomes, the generation of very high-molecular-weight species of SFKs upon ubiquitination indicates that these are multi and/or polyubiquitinated. Such a modification is known to be an efficient proteasomal degradation signal consistent with stabilization of SFKs by proteasome inhibitors (Figs. 1B and 3; refs. 28, 29). This distinct nature of SFK ubiquitination as compared with monoubiquitination of receptor tyrosine kinases and other membrane proteins is intriguing given that SFKs in their biologically active forms are myristoylated and/or palmitoylated, and therefore exclusively membrane anchored. Whether this ubiquitination might reflect the localization of SFKs in specialized glycosphingolipid-rich membrane microdomains (30) or the possible involvement of Ub chain elongation machinery are obvious questions that will need to be addressed. Notably, Cbl has been shown to translocate to lipid-rich membranes microdomains upon FcεRI ligation in mast cells (31). It also remains possible that alternate, proteasome-independent mechanisms exist to degrade SFKs.

Our studies also indicate that Cbl-dependent ubiquitination and degradation is specifically directed toward the active pool of Lck. The Cbl-Lck association was markedly induced by TCR/

CD4 coligation, consistent with the occlusion of Cbl-binding SH3 and SH2 domains in repressed SFKs. A similar activation-induced association between Cbl and other SFKs has been noted (32, 33). Furthermore, our analyses of Cbl<sup>-/-</sup> and Cbl<sup>+/+</sup> cells showed a clear accumulation of the kinase-active pool of Lck (Fig. 2). Finally, an activated mutant of Lck (Y505F) was more susceptible, whereas a kinase dead Lck (D273A) was resistant to Cbl-dependent ubiquitination and degradation compared with WT Cbl (Fig. 4B). A similar susceptibility of activated Fyn and Src to negative regulation by Cbl has emerged recently (11, 12). It is likely that the selectivity of Cbl toward activated SFKs reflects the critical role of SFK SH3 domains (Fig. 5) for physical association with Cbl (11), as well as the role of the Cbl TKB domain (Fig. 5) that was observed to bind to the phosphorylated activation loop of Src (20). This motif is fully conserved among most SFKs including Fyn and Lck. Although previous studies have demonstrated that Cbl can bind to the GST-SH2 domain of Lck and Fyn *in vitro* (24, 34), our data indicated a minimal role of the Lck SH2 domain in Lck-Cbl association under our experimental conditions (Fig. 5). Similar data have emerged with Cbl-Fyn interactions (A.G., N.R., and H.B., unpublished data).

Our demonstration of Lck as a direct target of Cbl-mediated negative regulation suggests a significant role of this interaction during T cell development. Although protein expression data are lacking, Cbl mRNA levels are highest in the thymus (15). Importantly, Lck is pivotal for T cell development, being involved in TCR-β allelic exclusion, thymocyte proliferation and positive selection (35). In this regard, it is notable that the Cbl<sup>-/-</sup> mice show enhanced positive selection of CD4<sup>+</sup> thymocytes (36). Studies with Lck transgenes have demonstrated that increased Lck kinase activity can enhance positive selection (37). It is therefore reasonable to postulate that accumulation of active Lck in Cbl<sup>-/-</sup> thymocytes may mediate the enhanced positive selection that has been described (36). Cbl<sup>-/-</sup> mice also exhibit increased cellularity in the thymus as well as peripheral lymphoid organs (36, 38). Our findings suggest that Cbl-mediated down-regulation of Lck, in addition to that of ZAP-70, could play a role in this observed phenotype.

Recent findings have implicated SFK ubiquitination in viral pathogenesis. For example, Winberg *et al.* demonstrated that the latent membrane protein 2A of Epstein-Barr virus enhances the ubiquitination of the SFK Lyn in B cells (28). Furthermore, the human papilloma virus E6 oncogene was shown to directly interact with the B cell-specific SFK Blk and induce its degradation through the HECT domain E3 ligase E6AP (39). At present, the role of Cbl in viral inactivation of SFKs remains unknown.

In conclusion, our results demonstrate that Cbl directly regulates the activated T cell-specific SFK Lck, by enhancing its ubiquitination and targeting it for subsequent degradation by the proteasome. Given the highly conserved structure among Cbl family members, the ability of Cbl to control the level of activated Lck suggest critical roles for Cbl family proteins in T cell development and function. Such roles are likely to be revealed when compound Cbl knockouts, such as Cbl<sup>-/-</sup>/Cbl-b<sup>-/-</sup> (which are lethal), are examined in the context of the T cell compartment.

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