

A Mouse with a Loss-of-function Mutation in the c-Cbl TKB Domain Shows Perturbed Thymocyte Signaling without Enhancing the Activity of the ZAP-70 Tyrosine Kinase

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Abstract

The unique tyrosine kinase binding (TKB) domain of Cbl targets phosphorylated tyrosines on activated protein tyrosine kinases (PTKs); this targeting is considered essential for Cbl proteins to negatively regulate PTKs. Here, a loss-of-function mutation (G304E) in the c-Cbl TKB domain, first identified in *Caenorhabditis elegans*, was introduced into a mouse and its effects in thymocytes and T cells were studied. In marked contrast to the c-Cbl knockout mouse, we found no evidence of enhanced activity of the ZAP-70 PTK in thymocytes from the TKB domain mutant mouse. This finding contradicts the accepted mechanism of c-Cbl-mediated negative regulation, which requires TKB domain targeting of phosphotyrosine 292 in ZAP-70. However, the TKB domain mutant mouse does show aspects of enhanced signaling that parallel those of the c-Cbl knockout mouse, but these involve the constitutive activation of Rac and not enhanced PTK activity. Furthermore, the enhanced signaling in CD4⁺CD8⁺ double positive thymocytes appears to be compensated by the selective down-regulation of CD3 on mature thymocytes and peripheral T cells from both strains of mutant c-Cbl mice.

Key words: CD3 • CD5 • T cell receptor • SH2 domain • Rac

Introduction

The development of mature T cells in the thymus occurs via selection processes that are regulated by the affinity of TCR for MHC-self-peptide complexes and the strength of downstream signals triggered by this interaction. If TCR engagement is absent, then immature thymocytes quickly die of neglect, whereas remaining thymocytes that receive signals undergo opposing fates of positive or negative selection. Those receiving strong signals from high affinity interactions with thymic ligands are actively deleted, whereas thymocytes expressing TCRs of lower affinity receive weaker signals that allow survival and differentiation into mature T cells. Therefore, the ability to generate functional T cells is dependent on precisely controlled mechanisms of ligand engagement and signal transduction. Key questions emerging from this requirement for precise control are the quantitative and qualitative identities of the signaling molecules that determine the strength of the selecting signal.

The strength of the response is initially determined by the affinity of the TCR for MHC-self-peptide complexes and the total number of receptor interactions that form after ligand engagement (for review see references 1, 2). A protein that has a key role in regulating both TCR numbers and the activity of downstream signaling events in thymocytes is c-Cbl (3–5). c-Cbl is a multi-adaptor proto-oncogene possessing E3 ubiquitin ligase activity by virtue of its RING finger domain, which recruits ubiquitin-conjugating enzymes (E2s) to signaling complexes. The major class of substrate for c-Cbl-directed polyubiquitylation is activated protein tyrosine kinases (PTKs).^{*} Activated PTKs are recognized by a unique and highly conserved region common to all Cbl proteins that is comprised of a variant SH2 domain, a calcium-binding EF hand and a four helix bundle (6). Because all three domains are required to target phosphotyrosines in PTKs, this region is collectively known as a tyrosine kinase binding (TKB) domain.

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^{*}Abbreviations used in this paper: DP, double positive; ES, embryonic stem; PTK, protein tyrosine kinase; SP, single positive; TKB, tyrosine kinase binding.

Analysis of CD4⁺CD8⁺ double positive (DP) thymocytes from *c-Cbl* knockout mice revealed a marked enhancement of TCR and CD3 levels (7, 8). This up-regulation appears to be independent of TCR triggering as TCR transgenic thymocytes maintained in a nonselecting, or MHC class II-deficient, environment also show elevated expression of the transgene TCR. This finding led to the conclusion that *c-Cbl* is required for antigen-independent down-regulation of the TCR-CD3 complex on the surface of thymocytes (8). Recent papers on this process in thymocytes and T cells (9, 10) also support the idea that *c-Cbl* functions by promoting degradation (and thereby opposing recycling) rather than enhancing ligand-induced internalization. Thymocytes from *c-Cbl*^{-/-} mice also show marked activation of the ZAP-70 PTK in response to anti-CD3 stimulation alone (7, 8). This is in contrast to wild-type thymocytes where ZAP-70 is unresponsive to this signal in the absence of costimulation of the CD4 receptor. The crucial role of CD4 cross-linking is to activate the Src family kinase Lck that phosphorylates ZAP-70 to trigger its kinase activity. Remarkably, however, in *c-Cbl*^{-/-} thymocytes ZAP-70 can be activated without detectable activation of Lck, suggesting that *c-Cbl* directly targets ZAP-70, and not its upstream activators (7). Furthermore, the enhanced activation of ZAP-70 in *c-Cbl*^{-/-} thymocytes is not solely due to increased TCR and CD3 levels because the ability of ZAP-70 to phosphorylate its substrates LAT and SLP-76 is maintained at times when ZAP-70 is down-regulated in wild-type thymocytes (11). Direct targeting of ZAP-70 is further implicated by studies showing an interaction between the *c-Cbl* TKB domain and a negative regulatory tyrosine in ZAP-70 at position 292 (12). These findings support the proposal by Naramura et al. (8) that *c-Cbl* is functioning at two levels: (a) to down-regulate the surface expression of TCR-CD3 complexes, and (b) to negatively regulate intracellular signaling by ZAP-70. The basic premise of this paper (i.e., *c-Cbl* is a negative regulator of ZAP-70) is based on these studies with *c-Cbl* knockout mice.

To better understand the mechanisms of these effects, we analyzed mice with a loss-of-function mutation in the *c-Cbl* TKB domain. This mutation was first identified in SLI-1, the *Cbl* orthologue in *Caenorhabditis elegans*, as a glycine to glutamic acid substitution at amino acid 315 (i.e., G315E) that restored vulval induction in worms with reduction-of-function mutations in the LET-23 receptor tyrosine kinase (13). These experiments were notable as they were the first to identify *Cbl* proteins as negative regulators of PTKs. Analyses of this mutation in mammalian cells have revealed that it abolishes both transformation of mouse fibroblasts by oncogenic forms of *c-Cbl* and the ability of the TKB domain to interact directly with activated PTKs (4). A well-studied example of this is the G306E substitution in the human *c-Cbl* TKB domain that abolishes its interaction with the negative regulatory tyrosine 292 in ZAP-70 (14). The molecular basis for this is suggested by structural studies showing that G306 lies near the universally conserved arginine (R294 in human

c-Cbl) present in all SH2 domains that hydrogen bonds with the phosphate group of the incoming tyrosine. A glutamic acid substitution at 306 could form a buried salt bridge with R294, thus preventing its interaction with phosphotyrosine (6).

In this paper, we extend the functional analysis of the TKB domain by producing mice with a G304E knockin mutation. The mouse TKB and SH2 domains exhibit 99.4% and 100% identity, respectively, to the human so the G306E and G304E mutations are identical. Unexpectedly, we found no evidence of enhanced ZAP-70 activity in the thymus of this mouse, but we did observe other similarities to the *c-Cbl* knockout mouse suggesting that signaling pathways independent of ZAP-70 are enhanced in both mutant mice.

Materials and Methods

Preparation of Targeting Construct. A 15-kb murine *c-Cbl* genomic clone isolated from a λUNI-ZAP 129 Sv library (7) was used to construct the G304E targeting vector. A 3.9-kb XbaI fragment subcloned into pBluescriptSK⁺ (Stratagene) was modified by site-directed mutagenesis using the primer 5'TGGGCTATTGAGTATGTTACTGC3'(G304E); the G→A mutation (underlined) creating a Gly→Glu substitution at amino acid 304. A 3.6-kb Sall-BamHI pGK-neomycin and HSV-thymidine kinase cassette flanked by *loxP* sites isolated from the pFlox vector (provided by P. Orban, EMBL, Heidelberg, Germany) was cloned upstream of the 3.9-kb XbaI G304E fragment. The targeting construct was completed by the addition of a 1.4-kb fragment upstream of the "floxed" pGKNeo/HSVTK cassette and a 4.0-kb fragment downstream of the 3.9-kb XbaI G304E segment, representing *c-Cbl* genomic sequences immediately 5' and 3' of the 3.9-kb XbaI fragment, respectively (see Fig. 1 A).

The resulting construct was released by restriction enzyme digestion with NotI and XhoI and electroporated into W9.5 129 Sv/J embryonic stem (ES) cells (15). Targeted ES cells were selected in 200 μg/ml G418, individual clones picked and expanded, then genomic DNA isolated and restricted with BamHI for analysis. ES cell clones that had correctly undergone homologous recombination were identified first by PCR using primers external and internal to the targeting construct respectively (external *c-Cbl* primer p1, 5'ACGATAGTCCCGTGGGAAGAGC3' [T-S204]; and internal *loxP* primer p2, 5'TGC-TATACGAAGTTATTAGGTCCCTCG3' [*loxP* rev]), and then confirmed by Southern blotting using a ³²P-labeled genomic probe (probe A, 0.8 kb) hybridizing outside the targeting construct (wild-type allele, 5.8 kb; targeted allele, 7.9 kb; see Fig. 1 A). Finally, Southern blotting using a probe to the *Neo* gene was used to identify clones that had undergone only a single integration event.

Generation of *c-Cbl*(G304E) Knockin Mice. Two correctly targeted G418-resistant ES cell clones were used to generate chimeric mice by microinjection into embryonic C57BL/6J blastocysts. Chimeras (85–100%) were mated with C57BL/6J mice, and a founder line was established from an agouti pup heterozygous for the G304E mutation. Heterozygous females were mated with C56BL/6 *Cre-deleter* transgenic male mice (16) to induce in vivo excision of the *loxP*-flanked pGKNeo and HSV-TK cassettes. Successful excision was demonstrated by Southern

blotting of HindIII-digested genomic DNA using a 0.8-kb probe B (detecting 11.0 vs. 7.2 kb before and after Cre deletion, respectively; see Fig. 1 A). The resultant “Cre-d” allele retains a single 34-bp *loxP* site as well as ~70-bp polylinker sequences incorporated during the construction of the targeting vector, allowing subsequent genotyping of mice by PCR on tail DNA using c-Cbl specific primers p3 (5′TTCTTAGCTCTCAATGTTTCTACTCTCC3′; 4.2RI) and p4 (5′CATGTAACCAGGGTGAGTTAC3′; VM269R) that flank the *loxP* site (PCR product from WT allele ~400 bp, from G304E allele ~290 bp; see Fig. 1 A). The presence of the G304E mutation in the targeted allele(s) was also confirmed by genomic sequencing. Heterozygous c-Cbl G304E knockin mice from Cre-*deleter* matings were mated together to establish breeding lines from which all mice used in these experiments were derived.

Thymocyte Stimulation and Preparation of Cell Lysates. Thymocytes were stimulated by incubation with biotinylated anti-CD3 (500A2), anti-CD4 (GK1.5) antibodies, and avidin cross-linking at 37°C as described previously (11). The cells were lysed in ice-cold *n*-octyl-β-D-glucopyranoside/NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA), 1 mM sodium orthovanadate, 0.2% NP-40, 60 mM *n*-octyl-β-D-glucopyranoside (Sigma-Aldrich), 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM NaF, and 1 μg/ml each of chymostatin, leupeptin, and pepstatin. After incubating for 10 min on ice, lysates were cleared by centrifugation at 1,500 g for 8 min.

Immunoprecipitation, Immunoblotting, and Rac GTP Assays. Cleared lysates were analyzed by immunoprecipitation and immunoblotting as described previously (11). Anti-Lck and MAPK antibodies were purchased from Santa Cruz Biotechnology, Inc., anti-ZAP-70 and c-Cbl antibodies from Transduction Laboratories, and anti-TCRζ from Zymed Laboratories. Polyclonal rabbit anti-ZAP-70 and mouse monoclonal antiphosphotyrosine (4G10) antibodies were provided by L. Samelson (National Institutes of Health) and B. Druker (Oregon Health and Science University, Portland, OR), respectively. Antibodies to Akt and phospho-Akt(Ser473), phospho-p42/44 MAPK(Thr202/Tyr204), SAPK/JNK, and phospho-SAPK/JNK(Thr183/Tyr185) were purchased from Cell Signaling Technology. Rac-GTP was precipitated from thymocytes lysed in 25 mM Hepes, pH 7.4, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, and 10% glycerol by incubation for 30 min at 4°C with agarose-bound GST-PAK-1-PBD (human PAK-1 amino acid residues 67–150; Upstate Biotechnology) followed by three washes with the lysis buffer. Rac1 was detected by immunoblotting with anti-Rac antibodies (Cat. No. R55620; Transduction Labs).

Flow Cytometry. Single cell suspensions from the thymus, bone marrow, spleen, and lymph node were stained with antibodies, and 10⁴ viable cells were analyzed on a Becton Dickinson FACSCalibur™ machine. Cells were collected using CELLQuest software (Becton Dickinson) and further analyzed using Flo Jo (Tree Star Inc.). The following antibodies were used: anti-CD3ε(145–2C11)-APC and -PE, (17A2)-PE, (500A2)-PE, anti-TCRβ(H57–597)-FITC and -PE, anti-CD4(RM4–5)-PE, anti-CD8(53–6.7)-FITC, anti-CD5(53–7.3)-APC, anti-CD11b(M1/70)-PE, anti-Ly-6G(Gr-1)(RB6–8C5)-FITC, and anti-CD69(H1.2F3)-PE (all from BD Biosciences). Cells were incubated with anti-CD16/CD32 (2.4G2) before staining to block Fc binding, and dead cells were gated by propidium iodide staining. To detect intracellular levels of ZAP-70, c-Cbl, Lck, and TCRζ, the cells were fixed and permeabilized using a Cytofix/Cytoperm kit (Becton Dickinson)

according to the manufacturer’s directions. After incubation with antibodies to the aforementioned proteins, the cells were washed extensively in media containing 0.1% saponin. Unlabeled antibodies were detected with APC-conjugated goat anti-mouse Ig (BD Biosciences). To detect intracellular levels of CD3ε, the cells were first incubated for two rounds with unlabeled 145–2C11 before fixing, permeabilizing, and staining with PE-labeled 145–2C11. To detect intracellular levels of TCRβ, the cells were first incubated for two rounds with FITC-labeled H57–597 before fixing, permeabilizing, and staining with PE-labeled H57–597.

Results

Generation of Mice with a Loss-of-Function Mutation in the c-Cbl TKB Domain. Mice with a Gly→Glu substitution at position 304 (i.e., G304E) were generated using 129 Sv/J-derived ES cells and the targeting construct outlined in Fig. 1 A. To induce deletion of the *loxP*-flanked neomycin cassette, heterozygous mice carrying the mutated *c-Cbl* gene were crossed to the Cre-*deleter* strain and the resulting mice were intercrossed to expand numbers for experimental use. Genomic DNA was PCR-amplified and sequenced to confirm the successful introduction of the G304E mutation (Fig. 1 A). Litters were genotyped by PCR analysis of genomic DNA extracted from tails at weaning, and the products obtained are shown in Fig. 1 A.

Mice homozygous for G304E c-Cbl (which we term E/E) were generally healthy, although significantly fewer than expected homozygous E/E offspring were produced from +/E matings (+/+ = 91, +/E = 154, and E/E = 31), suggesting that developmental defects are associated with this mutation.

Examination of organs and tissues revealed no gross abnormality except for slight splenomegaly and dilated uterine horns and fallopian tubes in 40% (6/15) of E/E females examined between the ages of 6–9 wk. The fallopian tubes and uterine horns showed minimal evidence of tissue hyperplasia, rather the expanded size appeared to be caused by obstruction to the distal genital tract, resulting in mucous retention. Spleens from the c-Cbl E/E and –/– mice were similar in several respects. Both were larger compared with normal littermates (E/E ≈ 50% and –/– ≈ 80%) and were darker in color due to an expansion of the red pulp (Fig. 1 B, top). In addition, E/E and c-Cbl –/– spleens had increased numbers of megakaryocytes, megakaryoblasts, and myelocytes, which were predominantly found in clusters toward the splenic periphery (Fig. 1 B). Analysis of bone marrow from E/E and knockout mice also revealed an expansion of cells in the myeloid lineage. This similarity between the two mutant mice was observed histologically (unpublished data) and by flow cytometry which showed increases in the proportion of CD11b (Mac-1) and Ly-6G (Gr-1/8C5) positive cells compared with normal littermates (Fig. 1 B, bottom). The increase in myeloid lineage cells was accompanied by an equivalent decrease in CD45R(B220) positive cells (unpublished data).

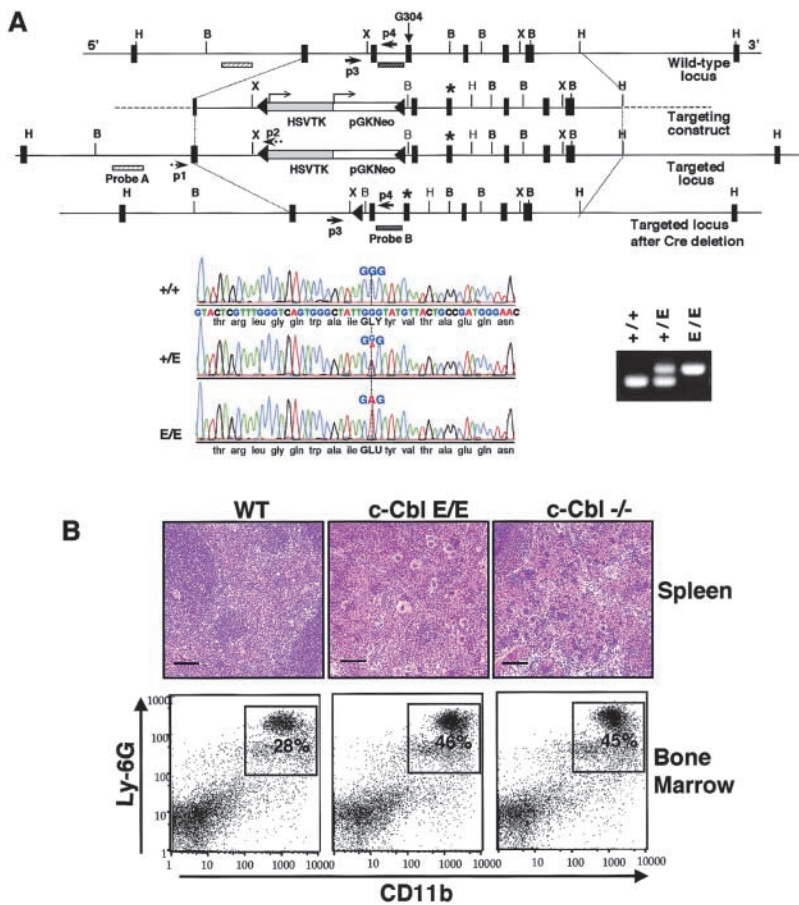


Figure 1. (A) Generation and identification of *c-Cbl*(G304E) mice. Genomic organization of the mouse *c-Cbl* gene (exons represented by black boxes) showing the relevant portion targeted for homologous recombination to introduce the G304E mutation (asterisk). Correctly targeted ES cell clones were identified by PCR using primers p1 and p2 (small dashed arrows), and confirmed by Southern blotting using probe A on BamHI-digested genomic DNA. The *loxP*-flanked Neo and TK cassettes were removed by Cre-mediated excision *in vivo*, leaving only a single *loxP* site (solid triangle). Excision was confirmed by Southern blotting of HindIII-digested genomic DNA using probe B. Genomic DNA sequenced across the G304 coding region confirmed the presence of the G→A nucleotide mutation in the +/E and E/E *c-Cbl* allele(s), resulting in the Gly→Glu amino acid change (bottom left). All mice were subsequently genotyped by PCR using primers p3 and p4 (small solid arrows) to detect the wild-type and G304E alleles (bottom right). B, BamHI; H, HindIII; X, XbaI. Restriction sites indicated in bold are present in the endogenous *c-Cbl* gene, whereas those in normal type were introduced during the construction of the targeting vector. (B) Top: Splenic sections from *c-Cbl*(G304E) and *c-Cbl*^{-/-} mice have increased numbers of megakaryocytes, megakaryoblasts, and myelocytes and an expansion in the red pulp. Spleen sections from 9-wk-old mice were stained with haematoxylin and eosin. Bar, 100 μ m. Bottom: Increase in bone marrow myeloid cells from *c-Cbl*(G304E) and *c-Cbl* knockout mice. Bone marrow cells from 8-wk-old mice were analyzed by flow cytometry for expression of CD11b(Mac-1) and Ly-6G (Gr-1/8C5) and the percentage of DP cells is indicated in each window. Equivalent increases in bone marrow myeloid lineage cells were found in four other experiments examining wild-type and mutant *c-Cbl* mice.

The G304E Mutation in c-Cbl Does Not Enhance ZAP-70 Activity in the Thymus. A hallmark of the *c-Cbl* knockout mouse is the enhanced and sustained activation of ZAP-70 in thymocytes after antibody cross-linking of CD3 and CD4 receptors (7, 8). This is evident from the very high levels of tyrosine phosphorylation of two prominent ZAP-70 substrates, SLP-76 and LAT (11). Furthermore, ZAP-70 activation in *c-Cbl* knockout thymocytes is uncoupled from a requirement for CD4-Lck costimulation. However, we did not find a similar response when we examined E/E thymocytes; indeed, the regulation of ZAP-70 activity appeared normal and paralleled that in wild-type thymocytes. This is illustrated in Fig. 2 A, where the tyrosine phosphorylation pattern clearly shows that ZAP-70 from E/E thymocytes could not be activated by anti-CD3 stimulation alone, and succeeding the stronger anti-CD3+CD4 costimulatory signal, the phosphorylation of ZAP-70, SLP-76, and LAT was similar to that of normal littermates. The marked contrast between the levels of LAT tyrosine phosphorylation in *c-Cbl*(G304E) and *c-Cbl*^{-/-} thymocytes was also shown by LAT immunoprecipitation (Fig. 2 B). The mutant *c-Cbl*(G304E) protein is expressed and tyrosine-phosphorylated to the equivalent levels of wild-type *c-Cbl* (Fig. 2 A). These findings indicate that *c-Cbl*(G304E) protein can still negatively regulate the activity of ZAP-70 in the thymus.

Thymocytes were additionally analyzed for effects of *c-Cbl* on MAP kinase and PI 3-kinase signaling pathways. Consistent with the effect on PTK activation, *c-Cbl* knockout thymocytes showed marked activation of Erk1, Erk2, and Akt as measured by immunoblotting with phosphospecific antibodies, whereas E/E thymocytes paralleled wild-type levels of activation (Fig. 2 C). In addition, unstimulated thymocytes from the *c-Cbl* knockout mouse showed higher levels of constitutively activated Erk1, Erk2, and Akt.

To more stringently examine the regulation of ZAP-70 by *c-Cbl*(G304E), we mated *c-Cbl*^{-/-} mice with E/E mice to discover whether heterozygous offspring with a single copy of *c-Cbl*(G304E) could rescue deregulated ZAP-70. As shown in Fig. 3 A, thymocytes with a single copy of the mutant allele (E/-) were able to regulate the tyrosine phosphorylation of ZAP-70, LAT, and SLP-76 as efficiently as thymocytes expressing two copies of wild-type *c-Cbl*. Furthermore, a single copy of *c-Cbl*(G304E) was sufficient to revert the thymocytes to their normal requirement for a CD4 costimulatory signal to activate ZAP-70 (α -CD3 stimulation; Fig. 3 B).

Rac Is Constitutively Activated in c-Cbl(G304E) and c-Cbl^{-/-} Thymocytes. There is increasing evidence that Cbl proteins can affect the organization of the actin cytoskeleton by regulating Rho family GTPases (for review see

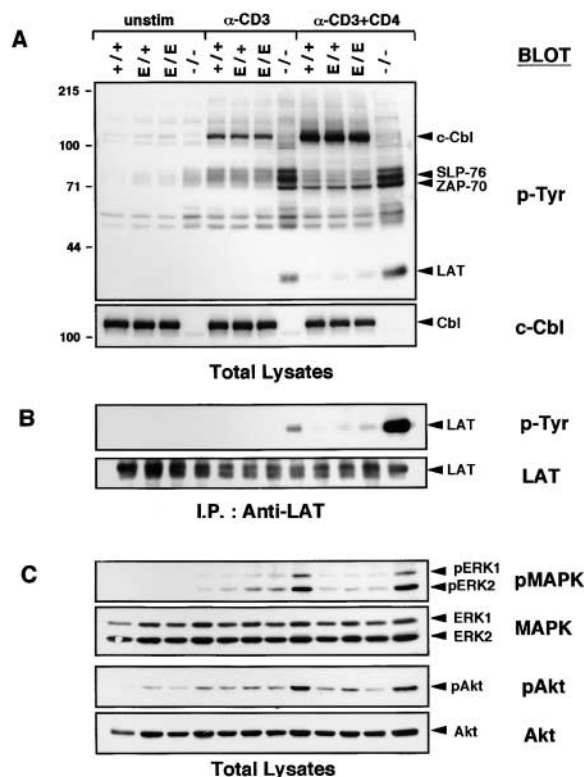


Figure 2. ZAP-70 regulation in activated thymocytes is not affected by the G304E mutation in the c-Cbl TKB domain. (A) Thymocytes from c-Cbl+/+, E/+, E/E, and -/- mice were left unstimulated or stimulated by antibody cross-linking at 37°C for 5 min before lysis. Total cell lysates were immunoblotted with antiphosphotyrosine and anti-c-Cbl antibodies. The positions of tyrosine-phosphorylated c-Cbl, SLP-76, ZAP-70, and LAT are indicated. (B) Thymocytes were treated as aforementioned, but lysates were immunoprecipitated with anti-LAT antibodies and immunoblotted with either antiphosphotyrosine or anti-LAT antibodies. (C) MAPK and Akt activation is not enhanced in c-Cbl(G304E) thymocytes. Thymocytes from c-Cbl+/+, E/+, E/E, and -/- mice were treated as in A. Total cell lysates were immunoblotted with phospho-p44/42 anti-Erk 1/2 and phospho-Akt antibodies. The levels of each protein were determined by immunoblotting with anti-Erk 1/2 and anti-Akt antibodies.

reference 4). The Rho family member Rac regulates cytoskeletal organization and TCR clustering, and is required for T cell maturation and the induction of T cell responses (for review see reference 17). By assaying the binding of Rac-GTP to the GST-p21-activated kinase binding domain (PAK-1 PBD), we found that unstimulated thymocytes from both c-Cbl(E/E) and c-Cbl-/- mice had markedly higher levels of Rac-GTP compared with wild-type thymocytes (Fig. 4). The constitutively enhanced Rac activity from both mutant mice was seen in five experiments and showed on average a three-fourfold increase in the level of Rac-GTP (Fig. 4 B). Interestingly, Rac-GTP levels were elevated in wild-type and c-Cbl(E/E) thymocytes after anti-CD3 stimulation, even though this signal does not activate ZAP-70. The highest levels of Rac-GTP were seen in c-Cbl knockout thymocytes; this effect is likely a consequence of ZAP-70 stimulation, which activates the guanine nucleotide exchange factor

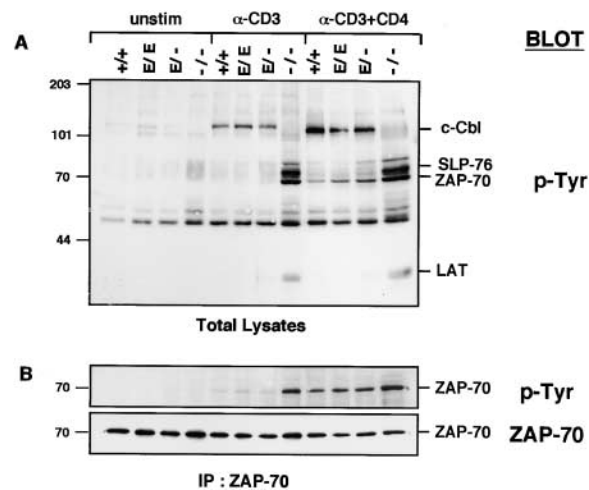


Figure 3. The enhanced activity of ZAP-70 in c-Cbl-/- thymocytes is suppressed by a single copy of c-Cbl(G304E). Thymocytes from c-Cbl+/+, E/E, E/-, and -/- mice were left unstimulated or stimulated by antibody cross-linking for 5 min at 37°C before lysis. (A) Total cell lysates immunoblotted with antiphosphotyrosine antibodies. The positions of tyrosine-phosphorylated c-Cbl, SLP-76, ZAP-70, and LAT are indicated. (B) Lysates were also immunoprecipitated with anti-ZAP-70 antibodies followed by immunoblotting with antiphosphotyrosine and anti-ZAP-70.

Vav1, leading to increased levels of Rac-GTP (for reviews see references 17, 18).

Regulation of Lck, ZAP-70, and TCRζ Levels in c-Cbl Mutant Mice. A prominent characteristic of c-Cbl knockout thymocytes is the up-regulation in the level of Lck (11). When we examined E/E thymocytes by immunoblotting (Fig. 5 A) and flow cytometry (Fig. 5 B), we found no increase in Lck above that of normal thymocytes. However,

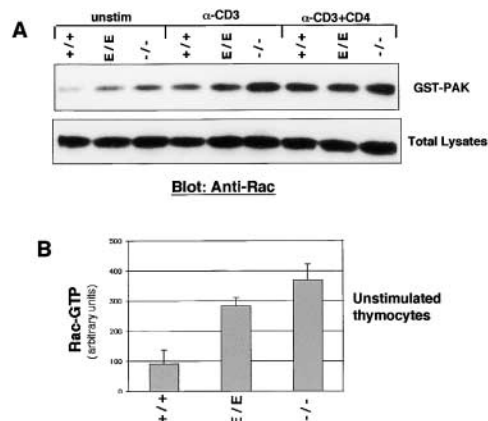


Figure 4. Rac is constitutively activated in thymocytes from c-Cbl(G304E) and knockout mice. (A) Lysates from unstimulated thymocytes or thymocytes stimulated by antibody cross-linking for 30 s at 37°C were incubated with agarose-bound GST-PAK-1 PBD. The levels of Rac-GTP bound to the Sepharose beads; in total, lysates were analyzed by immunoblotting with an anti-Rac antibody. (B) Densitometric scans for anti-Rac blots of GST-PAK-PBD-bound proteins from unstimulated thymocytes were obtained from five experiments and the averages plotted. Error bars represent standard deviations.

a consistent feature in *c-Cbl*^{-/-} and *E/E* thymii was a reduction in Lck protein levels after stimulation (Fig. 5 A). Whether this is a consequence of lowered antibody detection due to protein modification and/or enhanced degradation is unknown.

The ability to measure intracellular Lck by flow cytometry allowed us to compare levels in immature thymocytes, which express low levels of CD3 (i.e., CD3^{lo}), with mature thymocytes (CD3^{hi}). Analysis of these populations showed that the up-regulation of Lck in the knockout mouse is restricted to immature thymocytes (Fig. 5 C), with no observable difference compared with wild-type levels on maturation (Fig. 5 D). Interestingly, two color analysis of surface CD3 and intracellular *c-Cbl* in wild-type thymocytes showed no difference in *c-Cbl* expression between CD3^{lo} and CD3^{hi} thymocytes (unpublished data). This suggests that the wild-type levels of Lck in the mature *c-Cbl*^{-/-} thymocytes is not a result of their normal counterparts being unable to express high levels of *c-Cbl* protein. At present, we do not understand why *c-Cbl* does not appear to affect Lck levels in mature thymocytes.

We showed previously that ZAP-70 levels were unaltered in knockout thymocytes (11) and, as expected, ZAP-70 levels were also normal in *E/E* thymocytes (Fig. 5 A). Intracellular staining and flow cytometry also revealed that ZAP-70 levels were not altered in thymocytes

or peripheral T cells of either *c-Cbl* mutant mouse (unpublished data).

TCR ζ levels in *c-Cbl*^{-/-} thymocytes are also enhanced (11), which is consistent with a recent report showing that *c-Cbl* promotes TCR ζ ubiquitylation (19). To examine whether the TKB domain mutation can affect TCR ζ levels, we compared thymocytes and T cells from *c-Cbl* wild-type, *E/E*, and knockout mice (Fig. 5 E). Two color flow cytometry of CD3 ϵ (extracellular) and TCR ζ (intracellular) revealed that immature thymocytes (CD3^{lo}) from *E/E* mice express normal levels of TCR ζ . As with Lck, the up-regulation of TCR ζ in the knockout mouse was restricted to immature thymocytes (CD3^{lo}) because wild-type levels were found in both CD3^{hi} thymocytes and peripheral T cells (Fig. 5 E).

Down-regulation of CD3 on Mature Thymocytes and Peripheral T Cells from -Cbl^{-/-} and G304E Mutant Mice. The proportion of cells in the major thymic subsets as defined by CD4 and CD8 are normal in the *c-Cbl*^{-/-} mouse (7, 8), and here we find the *E/E* thymus to be similarly unaltered (unpublished data). However, CD4⁺CD8⁺ DP thymocytes from the *c-Cbl*^{-/-} mouse have markedly enhanced levels of CD3 and TCR (7, 8). In contrast, DP thymocytes from the *E/E* mouse showed no evidence of this enhancement because receptor levels were nearly equal, though not identical, to that of normal littermates

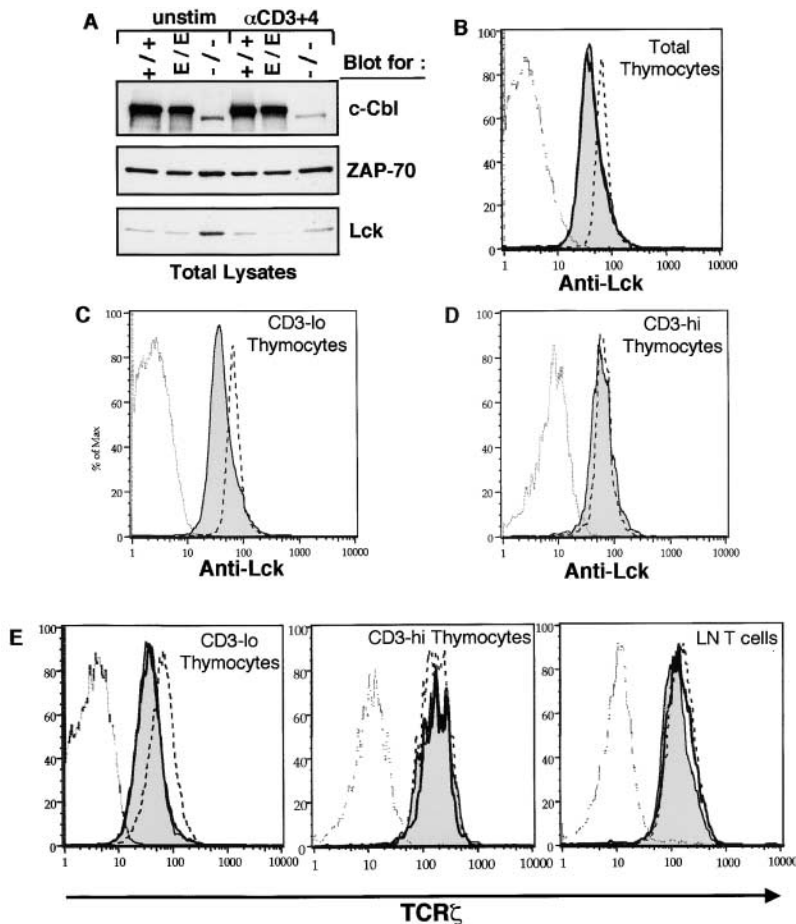


Figure 5. (A) Regulation of Lck and ZAP-70 levels in thymocytes from *c-Cbl* mutant mice. Thymocytes from *c-Cbl*^{+/+}, *E/E*, and ^{-/-} mice were left unstimulated or stimulated by antibody cross-linking for 5 min at 37°C and levels of *c-Cbl*, ZAP-70, and Lck determined by immunoblotting of total lysates. (B–D) Thymocytes were stained with anti-CD3-PE antibodies before fixing and intracellular staining with either anti-Lck monoclonal antibody or a mouse IgG isotype control. Antibody binding was detected with an APC-conjugated goat anti-mouse Ig, and the cells were analyzed by flow cytometry. (B) Lck staining of wild-type thymocytes (shaded histogram) and *E/E* thymocytes (bold line) showed identical overlapping profiles, whereas Lck levels in *c-Cbl*^{-/-} thymocytes are elevated (long dashes). The isotype antibody control for wild-type thymocytes is shown by small dashes. (C and D) Wild-type and *c-Cbl*^{-/-} thymocytes were gated for CD3^{lo} and CD3^{hi} populations, respectively, and intracellular staining for Lck is shown for wild type (shaded histograms) and *c-Cbl*^{-/-} (long dashes). The isotype antibody control for wild-type thymocytes is shown by small dashes. (E) TCR ζ levels in *c-Cbl* mutant mice. Thymocytes and lymph node cells were stained with PE-conjugated anti-CD3 antibodies before fixing and intracellular staining for TCR ζ . After flow cytometric analysis, thymocytes were gated into CD3^{lo} and CD3^{hi} populations, and lymph node cells were gated for CD3⁺ cells. Intracellular levels of TCR ζ are shown from wild-type (shaded histograms), *c-Cbl* *E/E* (bold line), and *c-Cbl*^{-/-} (long dashes) mice. The isotype antibody control for wild-type thymocytes is shown by small dashes and was identical to the isotype controls from mutant mice.

(Fig. 6, B and F, compare shaded histogram of wild type with bold line of E/E). We consistently found a minimal reduction in CD3 (Fig. 6 B) and a slight enhancement in TCR levels in E/E DP thymocytes (Fig. 6 F), but neither change was comparable in magnitude to the effect seen in the knockout.

A surprising but consistent feature of the anti-CD3 profiles was the reduced surface expression on mature thymocytes (i.e., CD3^{hi}) from both *c-Cbl*^{-/-} and E/E mice (Fig. 6 A, bright cells). This was readily apparent when we examined single positive (SP) CD4⁺ and CD8⁺ thymocytes and found both mutant strains to have equivalent reductions in CD3 levels compared with normal littermates

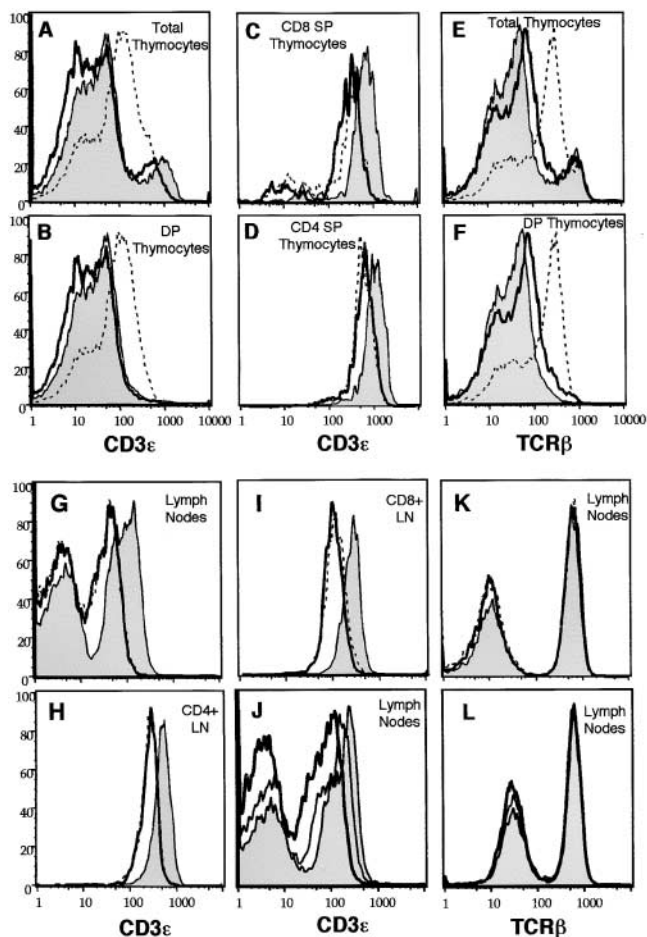


Figure 6. *c-Cbl*^{-/-} and G304E mutant mice show down-regulation of CD3ε on mature thymocytes and peripheral T cells. Ungated (Total thymocytes) or gated DP, CD4 SP, and CD8 SP thymocytes from wild-type (shaded histogram), *c-Cbl*(E/E) (bold line), and *c-Cbl*^{-/-} (dashed line) mice were analyzed by flow cytometry for cell-surface expression of CD3ε (A–D) and TCRβ (E and F). (G–I) Lymph node cells were analyzed for cell-surface expression of CD3ε, which is shown on ungated lymph node cells, CD4 SP, or CD8 SP T cells from wild-type (shaded histogram), *c-Cbl*(E/E) (bold line), and *c-Cbl*^{-/-} (dashed line) mice, respectively. TCRβ expression for this experiment is shown on ungated lymph node cells in K. (J and L) Lymph node cells from wild-type (shaded histogram), *c-Cbl*(E/E) (bold line), and heterozygous *c-Cbl*(+/E) (normal line) mice were analyzed for cell-surface expression of CD3ε and TCRβ, respectively.

(Fig. 6, C and D). This identity was seen for all E/E and ^{-/-} mice, even when we compared CD3 levels between different litters and different experiments. Thus, a strong selection pressure exists in *c-Cbl* mutant mice to express reduced levels of CD3 on mature thymocytes. This also indicates that the selection is precisely regulated and likely to involve a signaling perturbation that is common to both mutant strains.

A similar reduction in CD3 expression was also observed in peripheral T cells from spleens (unpublished data) and lymph nodes (Fig. 6 G) of *c-Cbl*^{-/-} and E/E mice, and seen in both CD4⁺ and CD8⁺ subsets (Fig. 6, H and I). The identical down-regulation of CD3 in both mutant mice was also evident with the 17A2 and 500A2 monoclonal antibodies (unpublished data), which recognize epitopes on CD3ε distinct to those recognized by the 145-2C11 antibody used in all CD3 experiments in this paper (20). Lymph node T cells from heterozygous +/E mice showed levels of CD3 intermediate to +/+ and E/E T cells (Fig. 6 J). Thus, the +/E mouse has a mild CD3 phenotype that correlates with gene dosage. This finding further indicates that *c-Cbl* can tightly regulate the level of CD3 that is ultimately expressed on selected mature T cells.

In contrast to CD3, no difference in TCR levels was seen on mature thymocytes (Fig. 6 E, TCR^{hi} cells) and lymph node T cells (Fig. 6, K and L) between normal littermates and mutant *c-Cbl* mice. This differential regulation of CD3 and TCR may be a consequence of the different roles played by these two classes of receptor; TCR is involved in antigen recognition but does not contribute to intracellular signaling, whereas CD3 components each contain an immune receptor tyrosine-based activation motif that is required to initiate downstream signaling events. Furthermore, in contrast to the TCR heterodimer, the CD3ε subunit contains endocytosis signals (21), although these signals are thought to contribute to the internalization and cell-surface down-regulation of the entire TCR–CD3 complex (9), rather than individual components as these data suggest.

To examine the regulation of CD3 in more detail, we compared mutant and wild-type mice for intracellular levels by incubating lymph node cells with unlabeled anti-CD3 antibodies to block cell-surface receptors followed by fixing, permeabilizing, and staining with anti-CD3-PE. Preincubation with unlabeled antibodies completely blocked surface staining with either PE or APC-labeled anti-CD3 antibodies (unpublished data). We found that the reduction of surface CD3 on lymph node T cells from *c-Cbl*^{-/-} and E/E mice (Fig. 7 A) was not paralleled intracellularly because all three mice showed equivalent levels of intracellular CD3 (Fig. 7 B). Thus, altered regulation by mutant *c-Cbl* specifically affects the amount of CD3 at the cell surface, not the intracellular pool. In contrast, both surface and intracellular levels of TCRβ are unaltered between wild-type and mutant T cells (Fig. 7, C and D).

Up-regulation of CD5 and CD69 in c-Cbl^{-/-} and G304E DP Thymocytes. CD5 is a cell-surface glycoprotein expressed on thymocytes, mature T cells, and a subset of B

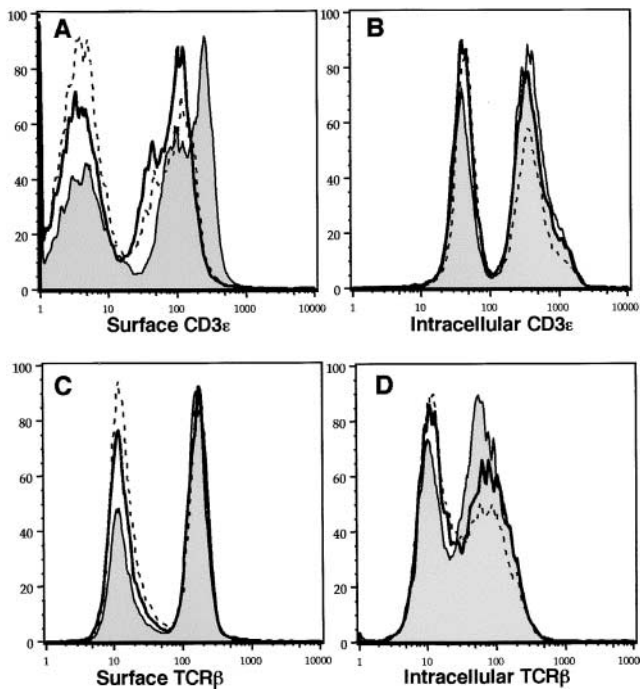


Figure 7. *c-Cbl*^{-/-} and G304E mutant mice show reduced surface, but not intracellular, CD3 ϵ levels in lymph node T cells. Lymph node cells were incubated with anti-CD3 ϵ -PE (A) or anti-TCR β -FITC (C) antibodies to determine cell-surface expression levels. To determine intracellular levels of CD3, the cells were first incubated with unlabeled anti-CD3 ϵ antibodies to block surface CD3 receptors, and then fixed, permeabilized, and stained with anti-CD3 ϵ -PE (B). Intracellular TCR β was determined by first blocking with FITC-labeled anti-TCR β , followed by fixing, permeabilizing, and staining with anti-TCR β -PE (D). Flow cytometry profiles show lymph node cells from 6-wk-old wild-type (shaded histogram), *c-Cbl*(E/E) (bold line), and *c-Cbl*^{-/-} (dashed line) mice. Pretreating the viable cells with unlabeled anti-CD3 or FITC-labeled anti-TCR was found to completely block these receptors from further surface staining (unpublished data).

cells that negatively regulates signaling through antigen receptors (22). CD5 expression on DP thymocytes has been found to parallel the affinity of the positively selecting TCR-MHC-ligand interaction, suggesting that its expression fine tunes the strength of the TCR-CD3 signaling response that selects the mature TCR repertoire (23). These observations are consistent with the up-regulation of CD5

seen in *c-Cbl* knockout DP thymocytes where the TCR-CD3 response is markedly enhanced (8). The G304E knock-in mouse also shows up-regulated CD5 expression on DP thymocytes, but the increase is less pronounced compared with knockout thymocytes (Fig. 8 A). Furthermore, when we compared DP thymocytes from *c-Cbl* +/E, E/E, E/-, and -/- mutant mice, we found sequential increases in CD5 that paralleled the severity of the mutation (Fig. 8 B). This effect indicates that perturbations in signal strength caused by mutant forms of *c-Cbl* are linked to, and finely tuned by, CD5 in a gene dosage-dependent manner. Up-regulation of CD5 in E/E DP thymocytes is additional evidence of enhanced signaling in this population, but this does not appear to involve deregulation of ZAP-70 activity.

CD69 is another marker whose expression is an indicator of thymocyte activation. It is induced on DP thymocytes within 24 h of productive TCR engagement by thymic ligands (24), and its up-regulation occurs during both positive and negative selection (25–27). As reported previously (8), DP thymocytes from the *c-Cbl* knockout mouse express higher levels of CD69 than those from wild-type mice (Fig. 8 C). As with CD5 expression, we found DP thymocytes from the E/E mouse also expressed elevated levels of CD69, but again the effect was not as marked as that seen for the knockout mouse. Analysis of mature thymocytes and lymph node T cells for levels of CD5 and CD69 revealed no differences between mutant and wild-type mice (unpublished data), further illustrating that *c-Cbl* effects on signaling appear to be limited to the DP population, whereas normal regulatory controls are restored in mature T cells.

Discussion

An unexpected outcome of our analysis of the *c-Cbl* (G304E) mouse is the finding that the *c-Cbl* TKB domain does not appear to be necessary for the negative regulation of ZAP-70 *in vivo*. This conclusion is drawn from the markedly different effects on ZAP-70 activation in thymocytes from the *c-Cbl* knockout compared with the *c-Cbl* (G304E) knockin mouse. At face value this finding contradicts a very large body of evidence showing that the

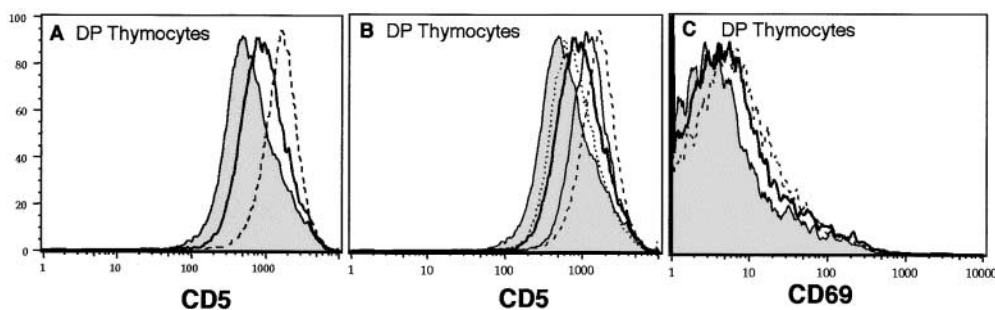


Figure 8. Up-regulation of CD5 and CD69 on thymocytes from *c-Cbl*^{-/-} and G304E mice. (A) DP thymocytes from 8-wk-old wild-type (shaded histogram), *c-Cbl*(E/E) (bold line), and *c-Cbl*^{-/-} (dashed line) mice were analyzed for cell-surface expression of CD5. (B) The same flow cytometry profiles as in A, except additional profiles from heterozygous +/E thymocytes (dotted line) and E/- (dashed line) mice were analyzed for cell-surface expression of CD5. (C) DP thymocytes from wild-type (shaded histogram), *c-Cbl*(E/E) (bold line), and *c-Cbl*^{-/-} (dashed line) mice were analyzed for cell-surface expression of CD69.

thymocytes (normal continuous line) are included. (C) DP thymocytes from wild-type (shaded histogram), *c-Cbl*(E/E) (bold line), and *c-Cbl*^{-/-} (dashed line) mice were analyzed for cell-surface expression of CD69.

c-Cbl TKB domain is essential for c-Cbl to negatively regulate ZAP-70. This was first implied by the demonstration of a direct interaction between the TKB domain and phosphorylated tyrosine 292 in ZAP-70 (28), previously identified as a negative regulatory site in ZAP-70 (29, 30). The interaction between ZAP-70 and the c-Cbl TKB domain has since been extensively characterized by an array of experimental systems and two structural studies (for reviews see references 4, 31). These all provide compelling evidence that c-Cbl functions as a negative regulator of ZAP-70 by TKB-domain targeting of phosphorylated tyrosine 292. However, such studies have principally been performed by transient transfection of Jurkat T cells, and none of these have clearly defined how the TKB domain is affecting the activity of ZAP-70. Furthermore, most studies have relied on NF-AT reporter gene expression as the means of measuring ZAP-70 function, so the direct effects of c-Cbl on upstream signaling proteins requires further investigation.

Consistent with there being no apparent effect of TKB domain mutation on ZAP-70 activity, we also found no phenotypic similarities to the ZAP-70(Y292F) mouse (32). The ZAP-70(Y292F) mouse does show a minimal overlapping phenotype with the c-Cbl knockout mouse in terms of increased LAT and TCR ζ tyrosine phosphorylation, but neither of these characteristics are seen in the G304E mouse. Furthermore, unlike the c-Cbl(G304E) mouse, DP and SP thymocytes from the ZAP-70(Y292F) mouse express normal levels of CD5 and CD3 ϵ , respectively. These findings indicate that even if the phenotypic effects seen in the c-Cbl(G304E) mouse are due to a presently unknown aspect of ZAP-70 function, they are clearly not mediated through phospho-Y292. In addition, the c-Cbl and ZAP-70 mutant mice do not show altered ZAP-70 protein levels, which indicates that c-Cbl, or another E3 ligase, does not target phospho-Y292 and direct ZAP-70 polyubiquitylation and degradation. Our comparison of c-Cbl knockout and G304E knockin mice has also clearly shown that it must be regions other than the TKB domain that are responsible for the negative regulation of ZAP-70, as well as controlling levels of TCR, CD3, and Lck in DP thymocytes. Therefore, it will be of interest to generate mice with mutations that affect regions outside the TKB domain, such as the RING finger and sequences involved in SH2 and SH3 domain interactions.

In this paper, the absence of an effect on ZAP-70 signaling by the G304E mutation was determined by examining the tyrosine phosphorylation of ZAP-70 and its substrates as well as the downstream activation of Erk and Akt. These findings suggested that signaling in G304E thymocytes is unaltered, and therefore should reveal phenotypic characteristics equivalent to that of wild-type mice. Surprisingly, we found some parameters were altered, and indeed paralleled the knockout mouse. One of these was the reduced level of CD3 ϵ on mature SP thymocytes and peripheral T cells. An identical decrease in cell-surface expression of CD3 ϵ was seen in both mutant strains, suggesting that the same signaling events are perturbed. Furthermore, we

found that the decrease in surface CD3 ϵ was not due to a global reduction in CD3 ϵ levels because an equivalent decrease was not found by staining for the intracellular pool of CD3 ϵ . Surprisingly, surface TCR β levels are unaffected by this reduction in surface CD3 ϵ . This was an unexpected finding because only fully formed TCR-CD3 hexameric complexes are thought to exit the endoplasmic reticulum and therefore TCR subunits should only be expressed on the cell surface as part of a complete complex (33). Furthermore, it has recently been shown that CD3 $\epsilon\epsilon$, CD3 $\gamma\gamma$, and CD3 $\delta\delta$ homodimers, as well as CD3 $\gamma\delta$ heterodimers, cannot exist (34), therefore it is unlikely that excess TCR on the surface is complexed with aberrant combinations of CD3 subunits. However, our data from c-Cbl mutant T cells does suggest that TCR $\alpha\beta$ chains are expressed on the cell surface in the absence of normal levels of CD3 ϵ subunits, and because no precedent exists for this phenotype, the mechanism requires further investigation.

From a functional perspective, we predict that reduced surface expression of CD3 is occurring to compensate for enhanced signaling in thymocytes undergoing selection, and the key task that emerges from this is to identify the nature of these signaling events. In the c-Cbl knockout mouse, the DP thymocytes exhibit numerous perturbations in the selecting population, i.e., increased levels of TCR β , TCR ζ , CD3 ϵ , and Lck, in addition to the altered regulation of ZAP-70, and these are prime candidates to explain the enhanced signaling observed after thymocyte stimulation (11). In spite of these perturbations, functionally normal T cells emerge in the periphery (7, 8). This is presumably because the knockout thymus has developed efficient mechanisms of compensation, and the selection of mature thymocytes with reduced surface CD3 levels is a likely component. In contrast, DP thymocytes from the c-Cbl(G304E) mouse show no increases in the levels of TCR β , TCR ζ , CD3 ϵ , and Lck, nor is there enhanced activity of ZAP-70, Erk, or Akt, although CD3 ϵ is down-regulated on selected thymocytes and T cells. From this, we conclude that the down-regulation of CD3 ϵ on selected T cells from the knockout mouse may in fact be a consequence of enhanced signaling that involves pathways unrelated to those associated with deregulated receptor levels and ZAP-70 activity described previously.

A characteristic that supports enhanced signaling in DP thymocytes from the G304E mouse is the increased expression of CD5 and CD69, both of which are indicators of thymocyte activation. The increase is not as marked as that seen in the knockout mouse, presumably because of additional contributions mediated by the up-regulation of TCR, CD3, Lck, and ZAP-70 in the knockout. However, identifying the signaling events in the G304E knockin mouse responsible for enhanced CD5 and CD69 expression, which necessitate the down-regulation of CD3 levels, is an important task.

We have identified the signaling candidate Rac, a member of the Rho family of GTPases that regulates actin reorganization in response to stimulatory signals and is required for the formation of the immunological synapse. Impor-

tantly, we found increased Rac-GTP levels in unstimulated thymocytes from both *c*-Cbl knockout and knockin mice. It may be that weak signals in the thymic environment are sufficiently enhanced at the level of Rac to promote excess actin-driven TCR-CD3 clustering, which sustains the signaling necessary for thymocyte activation. Indeed, the movement of the TCR-CD3 complex at the peak of ligand-induced signaling correlates with its linkage to actin (2), and a shift in the strength of this signal may be sufficient to alter the phenotype of selected thymocytes and T cells. Furthermore, it is significant that the CD3 ϵ subunit has recently been shown to be directly involved in this process via an activation-induced association with Nck, which functions as a scaffold to link signaling effectors involved in actin reorganization (35). Thus, CD3 ϵ is an essential signaling component in the formation of the immunological synapse, and its altered expression is predicted to affect downstream proteins such as Rac. Furthermore, an effect on cytoskeletal regulation in *c*-Cbl mutant mice would be consistent with effects seen in peripheral T cells from Cbl-b knockout mice. These mice revealed that Cbl-b functions as a negative regulator of TCR clustering by enhancing the activity of Vav1 and the Rho family member Cdc42 (36). However, these effects are restricted to peripheral T cells and are not evident in the thymus (37, 38). Indeed, analyses of Cbl-b knockout mice indicate that Cbl-b does not play a role in thymocyte signaling (37, 38), possibly because its levels are at least fivefold lower than those of *c*-Cbl (unpublished data). However, perturbations at the thymocyte level may be less detrimental for *c*-Cbl mutant mice as they have the opportunity to make the necessary adjustments to produce functionally normal T cells, thus avoiding autoimmune disease that develops in Cbl-b-deficient mice (37, 38).

The analysis of a mouse with a mutation that was originally described in *C. elegans* also raises the question of how these findings help to explain the rescue of a reduction-of-function mutation in the LET-23 receptor tyrosine kinase. At this point, the results may be helpful in directing analyses toward the possibility that the rescue may involve enhanced signaling at the level of a Rho family member, rather than a direct effect on LET-23 or other PTKs. The lack of evidence of enhanced ZAP-70 signaling in Cbl(G304E) thymocytes indicates that this is a possibility worthy of consideration.

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