A direct interaction between the adaptor protein Cbl-b and the kinase Zap-70 induces a positive signal in T cells

Zhihong Zhang, Chris Elly, Ling Qiu, Amnon Altman and Yun-Cai Liu

Engagement of the T-cell receptor (TCR)–CD3 complex induces a rapid increase in the activities of Src-family and Syk/Zap-70-family kinases [1,2]. These activated kinases then induce the tyrosine phosphorylation of multiple intracellular proteins, eventually leading to T-cell activation. One of the prominent substrates for these kinases is the adaptor protein Cbl [3] and recent studies suggest that Cbl negatively regulates upstream kinases such as Syk and Zap-70 [4,5]. Cbl-b, a homologue of Cbl, is widely expressed in many tissues and cells including hematopoietic cells [6,7]. Cbl-b undergoes rapid tyrosine phosphorylation upon stimulation of the TCR and cytokine receptors [8,9]. The role of Cbl-b is unclear, however. Here, we show that overexpression of Cbl-b in T cells induced the constitutive activation of the transcription factor nuclear factor of activated T cells (NFAT). A loss-of-function mutation in Cbl-b disrupted the interaction between Cbl-b and Zap-70 and nearly completely abrogated the Cbl-b-mediated activation of NFAT. Unlike the proposed role of Cbl as a negative regulator, our results suggest that the Cbl homologue Cbl-b has a positive role in T-cell signaling, most likely via a direct interaction with the upstream kinase Zap-70.

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Results and discussion

To begin to understand the functional involvement of Cbl-b in T-cell signaling, we assessed the effect of transiently overexpressed Cbl-b on transactivation of a luciferase reporter gene driven by NFAT-binding sites (NFAT–luc). This NFAT transcriptional unit is derived from the interleukin-2 (IL-2) gene [10,11] and has been widely used as an indicator for T-cell activation. Lysates of human leukemic Jurkat T cells expressing SV40 large T antigen (Jurkat-TAg cells) that had been transiently transfected with either empty vector or a plasmid encoding Cbl-b together with the NFAT–luc reporter plasmid were assayed for luciferase activity under different stimulation conditions. Overexpression of Cbl-b induced NFAT activation under resting conditions and this was enhanced upon stimulation with anti-CD3 antibody (Figure 1a). A synergy between Cbl-b and a TCR-mediated signal was observed for the induction of NFAT activation when different amounts of anti-CD3 antibody were used for stimulation (Figure 1b). In contrast, overexpression of Cbl inhibited both basal and anti-CD3-stimulated NFAT activation under the same conditions. Normalization of the luciferase activity from the reporter in these experiments relative to the maximal luciferase activity induced by phorbol myristate acetate (PMA) and ionomycin or to a reference β-galactosidase activity showed similar results (see Supplementary material published with this paper on the internet). The level of Cbl-b-mediated activation of NFAT correlated with its protein expression level, as shown by titration of the plasmid amounts for transfection. Even low levels of Cbl-b induced activation of NFAT under basal and anti-CD3-stimulated conditions (Figure 1c).

Recent studies suggest that Cbl-family proteins participate in the regulation of the protein tyrosine kinases with which they associate [4]. We recently demonstrated that Cbl-b binds Syk and that a loss-of-function mutation (a glycine to glutamic acid substitution at position 298, G298E) in Cbl-b disrupts its interaction with Syk [9]. We then analyzed the effect of the G298E mutation on NFAT activation. Overexpression of the G298E Cbl-b mutant completely abolished Cbl-b-induced NFAT activation under basal and ionomycin-stimulated conditions (Figure 2a). A large reduction in NFAT activity induced by this mutant compared with that induced by wild-type Cbl-b was observed under anti-CD3-stimulated conditions.

To further confirm that the G298E mutation in Cbl-b achieved its effect by disrupting the interaction between Cbl-b and Zap-70, we then examined the molecular basis of Cbl-b–Zap-70 binding. Coexpression of Cbl-b with Zap-70 caused basal-level tyrosine phosphorylation of Cbl-b, which was increased upon anti-CD3-stimulation (Figure 2b). In contrast, the G298E Cbl-b mutant was not tyrosine phosphorylated under resting conditions. Anti-CD3 stimulation induced only a low level of tyrosine phosphorylation of this mutant. Zap-70 coimmunoprecipitated with Cbl-b, and this interaction was enhanced by anti-CD3 stimulation. A markedly reduced amount of Zap-70 coimmunoprecipitated with the G298E Cbl-b mutant, however (Figure 2b).

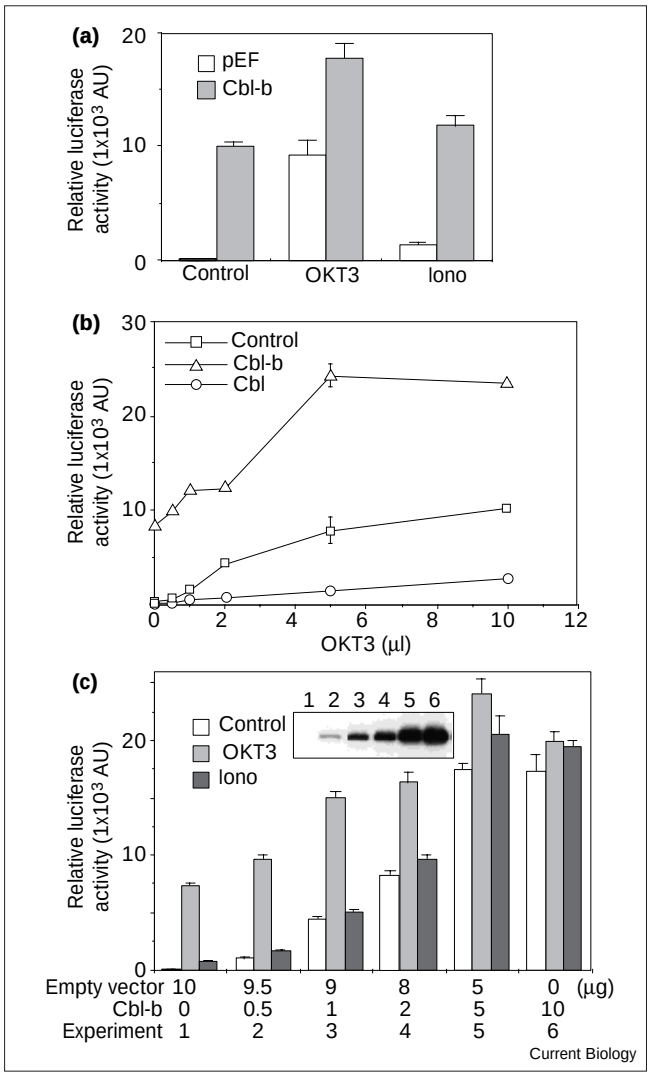


Figure 1

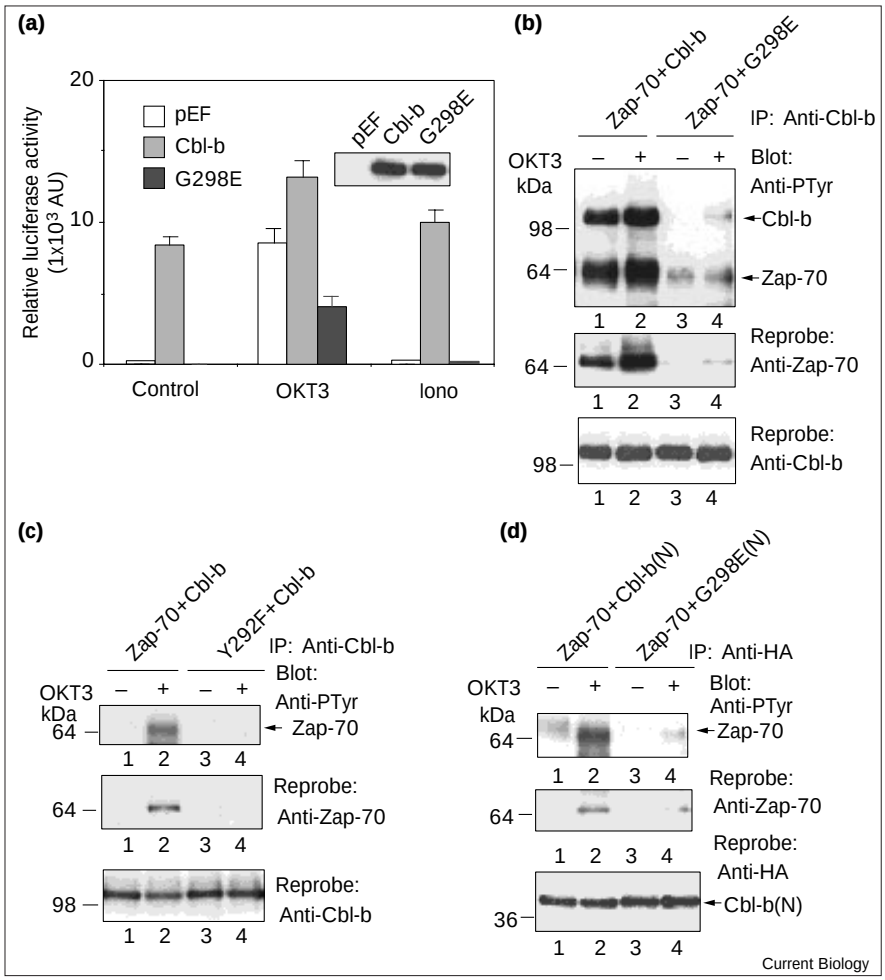
Effect of Cbl-b on NFAT induction in Jurkat T cells. (**a**) Jurkat-TAg cells were transfected with empty vector (pEF) or a plasmid encoding hemagglutinin (HA)-tagged full-length Cbl-b (5 μg each) plus 5 μg NFAT–luc reporter plasmid. At 24 h after transfection, cells were either left unstimulated (control), or stimulated with anti-CD3 (OKT3) ascites (1:500), or with ionomycin (iono; 100 ng/ml) for 6–10 h. Cells were then lysed and the luciferase activity was determined as described previously and expressed in arbitrary units (AU) [17]. (**b**) Cells were transfected with empty vector (control), plasmids encoding Cbl-b or Cbl (5 μg each) plus 5 μg NFAT–luc reporter plasmid. At 24 h after transfection, cells were either left unstimulated or stimulated with different amounts of anti-CD3 antibody (OKT3; μl) at a dilution of 1:10 for 6–10 h. Lysates were then assayed for luciferase activity. (**c**) Jurkat-TAg cells were transfected with different amounts of plasmid encoding HA-tagged Cbl-b plus an equal amount of NFAT–luc reporter gene (5 μg in each case). The total amounts of plasmids used for each transfection were adjusted to the same final amount by addition of empty vector where necessary. At 24 h after transfection, cells were either left unstimulated (control), or stimulated with anti-CD3 (OKT3) ascites (1:500), or with ionomycin (iono; 100 ng/ml) for 6–10 h. The luciferase activity induced by PMA plus ionomycin was similar among all the groups (data not shown). The insert shows aliquots of cell lysates from each transfection experiment immunoblotted with anti-HA antibody to detect the levels of Cbl-b protein. The results shown in (a–c) are representative of at least six separate experiments. Bars represent the means of triplicate samples. Standard deviations are depicted by error bars.

Both Cbl and Cbl-b interact with Tyr316 of Syk [9,12], suggesting that these two molecules bind to the same site in Syk. As Cbl binds to the corresponding tyrosine residue of Zap-70 (Tyr292) [13], we then examined whether Tyr292 in Zap-70 was also required for the interaction with Cbl-b. To this end, Jurkat-TAg cells were transfected with plasmids expressing wild-type Zap-70 or a Zap-70 mutant containing a tyrosine to phenylalanine substitution at position 292 (Y292F) together with a plasmid encoding Cbl-b. Consistent with the data in Figure 2b, Zap-70 was coimmunoprecipitated from cells expressing wild-type Zap-70 and Cbl-b (Figure 2c). A markedly reduced amount of the Y292F Zap-70 mutant coimmunoprecipitated with Cbl-b under both resting and OKT3-stimulated conditions, however.

We recently showed that Syk binds to the amino-terminal phosphotyrosine-binding (PTB)-like domain of Cbl-b [9]. To assess whether this is also true for the Zap-70–Cbl-b interaction, Jurkat-TAg cells were cotransfected with a plasmid encoding Zap-70 together with a plasmid encoding the amino-terminal 349 amino acids of Cbl-b, Cbl-b(N), or a mutant form of this fragment containing the G298E mutation. Cell lysates were immunoprecipitated with anti-Cbl-b antibody and then immunoblotted with anti-phosphotyrosine antibody. As demonstrated for full-length Cbl-b and the G298E Cbl-b mutant (Figure 2b), the form of Cbl-b(N) containing the G298E mutation did not interact with Zap-70 (Figure 2d), indicating that the observed Zap-70–Cbl-b interaction is mediated via the amino-terminal portion of Cbl-b.

The similar interaction between full-length Cbl-b or Cbl-b(N) and Zap-70 prompted us to assess a functional role for Cbl-b(N) in NFAT activation. Unlike full-length Cbl-b, Cbl-b(N) did not induce NFAT activation under resting conditions (Figure 3a), and in fact decreased NFAT activation under anti-CD3-stimulated conditions. This result implies that, by binding to Zap-70, Cbl-b(N) may play a dominant-negative role in events downstream of Cbl-b. To test this hypothesis, we cotransfected a plasmid encoding Cbl-b with plasmids encoding Cbl-b(N) or the G298E Cbl-b(N) mutant and the NFAT–luc reporter plasmid, and assessed their respective effects on NFAT activation. As shown in Figure 3b, coexpression of Cbl-b with Cbl-b(N) inhibited Cbl-b-induced NFAT activation. This inhibitory effect was largely rescued by coexpression of the G298E Cbl-b(N) mutant. Titration of the plasmid amounts showed that even as little as 0.5 μg Cbl-b(N) plasmid (that is, a Cbl-b(N) : Cbl-b ratio of 1:4) displayed an inhibitory effect on Cbl-b-mediated NFAT activation. This result suggests that Cbl-b(N) exerts its

Figure 2



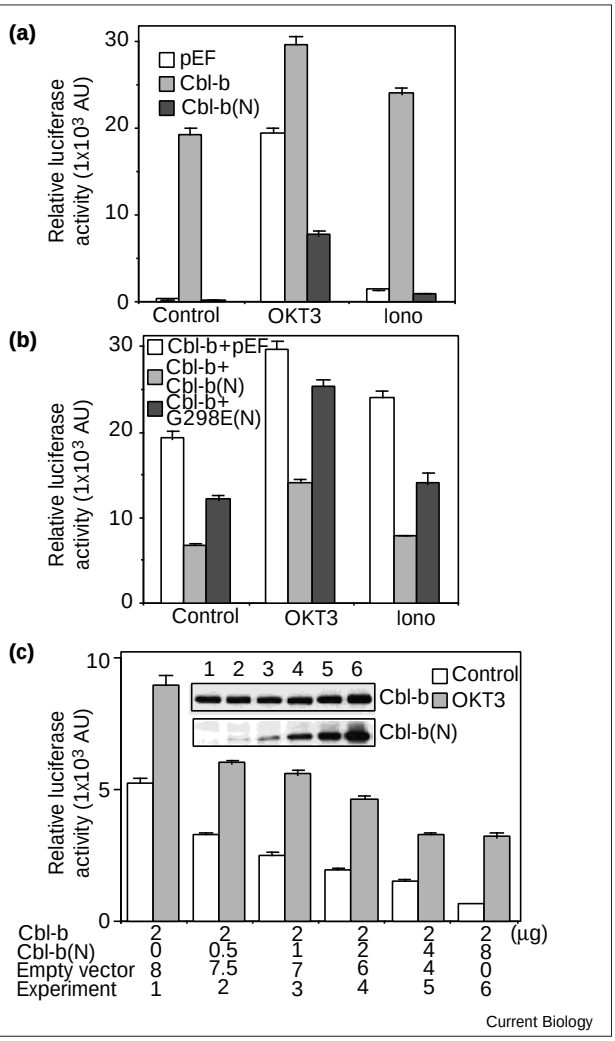
Effect of the G298E mutation in Cbl-b on Cbl-b-mediated NFAT activation and on the interaction between Cbl-b and Zap-70. (a) Jurkat-TAg cells were transfected with the NFAT–luc reporter plasmid (5 μg) plus either empty vector (pEF), or plasmids encoding HA-tagged Cbl-b or G298E Cbl-b mutant (G298E; 5 μg each). At 24 h after transfection, cells were either left unstimulated (control), or stimulated with anti-CD3 (OKT3) ascites (1:500), or with ionomycin (iono; 100 ng/ml) for 6–10 h. Cell lysates were analyzed for luciferase activity. Bars represent the mean of triplicate samples. The data shown are representative of three independent experiments. Aliquots of cell lysates were analyzed with anti-HA antibody to detect levels of Cbl-b expression (insert). (b) Jurkat-TAg cells were transfected with plasmid containing Zap-70 (2 μg) together with plasmid containing Cbl-b or G298E Cbl-b (5 μg each). At 24 h after transfection, lysates were prepared from unstimulated (–) or anti-CD3-stimulated (+; 5 min) cells, immunoprecipitated with anti-Cbl-b antibody, and immunoblotted with anti-phosphotyrosine antibody (anti-PTyr), according to our published protocol [18]. The positions of Cbl-b and Zap-70 are indicated by arrows. The membrane was reprobed with anti-Zap-70 antibody (middle panel), then with anti-Cbl-b antibody (lower panel). (c) Jurkat-TAg cells were transfected with plasmid encoding Cbl-b together with plasmid containing Zap-70 or the Y292F Zap-70 mutant (Y292F). Cell lysates prepared as in (b) were immunoprecipitated with anti-Cbl-b antibody and immunoblotted with anti-phosphotyrosine antibody (upper panel). The position of Zap-70 is indicated by an arrow. The membrane was reprobed with anti-Zap-70 antibody (middle panel), then with anti-Cbl-b antibody (lower panel). (d) Jurkat-TAg cells were transfected with Zap-70 plasmid (2 μg) plus empty vector, and a plasmid containing an HA-tagged form of the amino-terminal region of Cbl-b (Cbl-b(N)), or a loss-of-function mutant of this fragment (G298E(N); 5 μg each). At 24 h after transfection, lysates were prepared from resting (–) or anti-CD3-stimulated (+; 5 min) cells, immunoprecipitated with anti-HA antibody and immunoblotted with anti-phosphotyrosine antibody (upper panel). The position of Zap-70 is indicated by an arrow. The same membrane was reprobed with anti-Zap-70 antibody (middle panel), then with anti-HA antibody (lower panel). The position of Cbl-b(N) is indicated by the arrow.

dominant-negative role by a direct, competitive interaction with Zap-70.

In summary, we demonstrate that overexpression of Cbl-b induces the constitutive transactivation of NFAT, which is further enhanced by anti-CD3 stimulation. This effect is mediated by a direct interaction between the Cbl-b PTB domain and the Tyr292 residue of Zap-70, as a loss-of-function mutation (G298E) in Cbl-b disrupted its interaction with Zap-70 and abolished Cbl-b-induced NFAT activation.

Accumulating evidence suggests that Cbl is a negative regulator of intracellular signaling: Cbl-deficient mice display lymphoid hyperplasia, and thymocytes from these mice exhibit elevated levels of Zap-70 kinase activity [5]; Cbl directly inhibits the kinase activity of Syk [4], and the TCR-induced activation of NFAT (Figure 1) and AP-1 [14]; and Cbl is involved in the hyporesponsiveness of anergic T cells [15]. Although the Cbl–Grb2 interaction [14] or the Cbl–Crk-L–Rap-1 signaling pathway [15] are thought to be responsible for the negative role of Cbl, a direct interaction between Cbl and Zap-70 [5] and/or Syk [4] most likely accounts for the inhibitory function of Cbl. In contrast, we show here that Cbl-b positively regulates T-cell signaling leading to NFAT activation, and that this is mediated by a direct interaction between Cbl-b and the Tyr292 residue of Zap-70. These results suggest that by binding to the same phosphorylated tyrosine residue of Zap-70, Cbl or Cbl-b can induce either a negative (for Cbl) or a positive (for Cbl-b) signal via Zap-70. Considering the critical role of Syk/Zap-70-family protein tyrosine

Figure 3



Effect of Cbl-b(N) on Cbl-b-induced NFAT activation. (a) Jurkat-TAg cells were transfected with the NFAT–luc reporter plasmid (5 μg) together with empty vector (pEF), or plasmids expressing full-length Cbl-b or Cbl-b(N) (5 μg each). At 24 h after transfection, cells were left unstimulated (control) or stimulated with anti-CD3 antibody (OKT3) or ionomycin (iono), as indicated, for 6–10 h. Cell lysates were analyzed for luciferase activity. Total amounts of plasmids were adjusted to 15 μg with empty vector. (b) Jurkat-TAg cells were transfected with a plasmid encoding Cbl-b (5 μg) together with empty vector (pEF), or plasmids encoding Cbl-b(N) or the G298E Cbl-b(N) mutant (G298E(N); 5 μg each) plus 5 μg NFAT–luc reporter plasmid. At 24 h after transfection, cells were either left unstimulated (control) or stimulated, as indicated, for 6–10 h and then assayed for luciferase activity. (c) Jurkat-TAg cells were transfected with plasmid encoding Cbl-b plus NFAT–luc reporter plasmid together with different amounts of Cbl-b(N) as indicated. At 24 h after transfection, cells were either left unstimulated (control) or stimulated with anti-CD3 (OKT3) ascites (1:500) for 6–10 h. Aliquots of cell lysates were analyzed with anti-HA antibody (insert) to detect protein levels of wild-type Cbl-b and Cbl-b(N). The data in (a–c) are representative of three independent experiments.

kinases in lymphocyte development and activation [16], it can be envisioned that a delicate functional balance between Cbl and Cbl-b may have a significant impact on these kinase-mediated immune responses.

Supplementary material

Additional data on the specificity of Cbl-b in NFAT activation and additional methodological detail are published with this paper on the internet.

Acknowledgements

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Supplementary material

A direct interaction between the adaptor protein Cbl-b and the kinase Zap-70 induces a positive signal in T cells

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Supplementary results

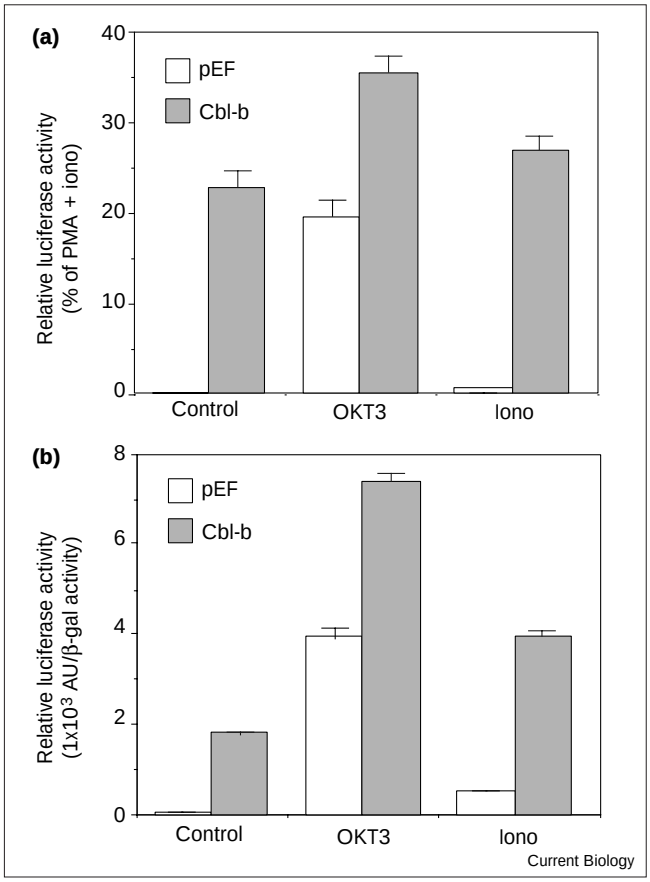
To ensure that the observed NFAT activation by Cbl-b was not a non-specific effect due to Cbl-b overexpression, we normalized the luciferase activity in relation to that induced by a combination of PMA and ionomycin stimulation. Under our experimental conditions, the maximal luciferase activity induced by PMA and ionomycin was normally about five times higher than that induced by anti-CD3 stimulation and no significant difference among different transfections was observed. As shown in Figure S1a, a similar pattern of the relative luciferase activity was observed, when normalized to the luciferase activity induced by PMA and ionomycin. As a further control, we cotransfected a plasmid containing β-galactosidase and the NFAT–luc reporter plasmid and empty vector or Cbl-b plasmid. When the luciferase activity was normalized to β-galactosidase activity, Cbl-b also induced a high level of NFAT activation under resting conditions that was enhanced by anti-CD3 stimulation (Figure S1b). It should be mentioned that coexpression with β-galactosidase caused a marked reduction in NFAT activity (~80%) (compare Figure S1b with Figure 1a), probably due to the interference of the β-galactosidase enzyme. Due to the similarity of all three methods, in the following experiments we adopted a simple method as in Figure 1a to present our luciferase data.

The IL-2-gene-derived NFAT transcriptional unit used in this study contains a binding site for the NFAT transcription factor and a binding site for AP-1 [S1,S2]. The integration of a Ca^{2+}-dependent and a Ras-dependent signal induces the transactivation of the promoter of the NFAT gene [S3–S5]. Next, we examined whether blockage of one of the two signals has any effect on Cbl-b-induced NFAT activation. As shown in Figure S2a, pretreatment of transfected cells with cyclosporin A (CsA), an inhibitor of calcineurin, reduced Cbl-b-induced NFAT activation under both basal and anti-CD3-stimulated conditions. Similarly, coexpression of a dominant-negative version of Ras, RasN17, almost completely abolished Cbl-b-induced NFAT activation under either resting or anti-CD3-stimulated conditions (Figure S2b). This effect of RasN17 did not reflect a non-specific inhibition of Cbl-b protein expression, as equivalent amounts of Cbl-b proteins were detected in the samples cotransfected with either the empty vector or plasmid encoding RasN17 (Figure S2c).

Supplementary discussion

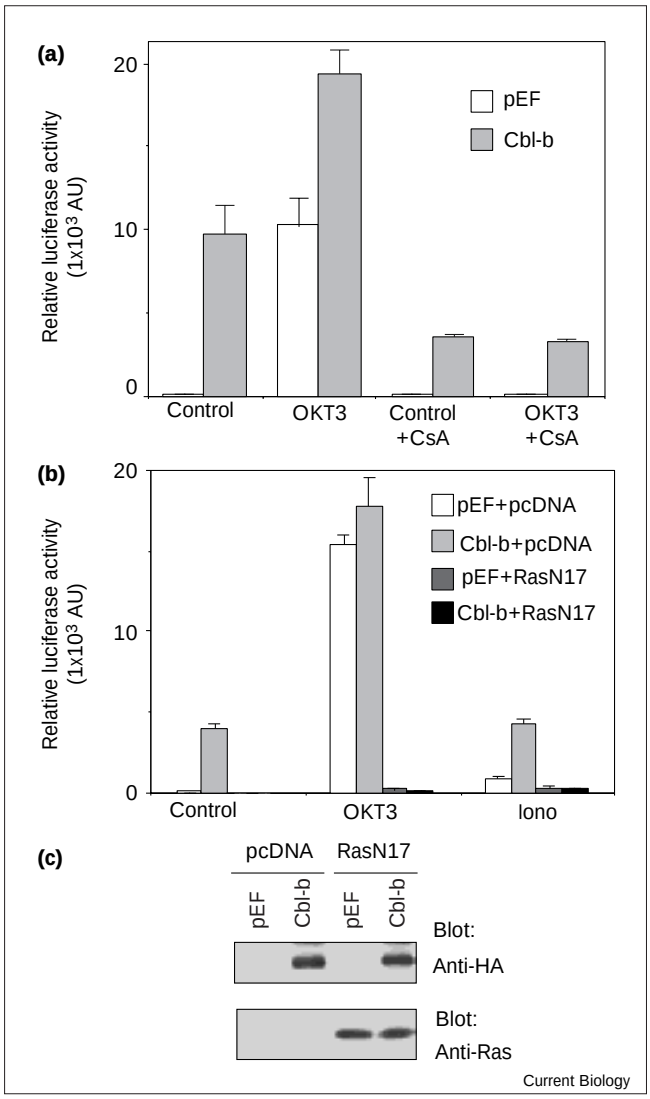
In the present study, we have demonstrated that overexpression of Cbl-b induced constitutive transactivation of NFAT, which was enhanced by anti-CD3 stimulation. This Cbl-b-mediated effect requires a Ca^{2+} signal and functional Ras, implying that the target of Cbl-b lies upstream of TCR-mediated signaling pathways. We

Figure S1



Effects of Cbl-b on NFAT induction in Jurkat T cells. (a) Jurkat-TAg cells were transfected with empty vector (pEF) or plasmids encoding HA-tagged full-length Cbl-b (5 μg each) plus 5 μg NFAT–luc reporter plasmid. At 24 h after transfection, cells were left unstimulated (control), or stimulated with anti-CD3 (OKT3) ascites (1:500), ionomycin (iono; 100 ng/ml), or PMA (50 ng/ml) plus ionomycin (100 ng/ml) for 6–10 h. Cells were then lysed and the luciferase activity was determined. The relative luciferase activity was converted in relation to the maximal activity induced by PMA plus ionomycin. (b) Cells were transfected as in (a) but with an additional reference reporter plasmid encoding β-galactosidase (3 μg). The relative luciferase activity was represented relative to β-galactosidase enzymatic activity. Bars represent the mean of triplicate samples. Standard deviations are depicted by error bars. The results shown in (a) are representative of three separate experiments.

Figure S2



Effects of cyclosporin A and a dominant-negative Ras on Cbl-b-induced NFAT activation. (a) Jurkat-TAg cells were transfected with empty vector (pEF) or plasmid encoding Cbl-b (5 μg each) plus NFAT–luc reporter plasmid. Cells were left untreated or treated with cyclosporin A (CsA, 50 ng/ml) for 10 min before treatment with or without anti-CD3 (OKT3) ascites (1:500), or ionomycin (iono; 100 ng/ml) for another 8–10 h. Luciferase activity was then determined. (b) Jurkat-TAg cells transfected with NFAT–luc reporter plasmid (5 μg) and either empty vector (pEF) or plasmid containing Cbl-b (5 μg each) plus a plasmid encoding dominant-negative Ras (RasN17) or a corresponding empty vector (pcDNA3; 5 μg each) were left unstimulated (control) or stimulated with anti-CD3 (OKT3), or ionomycin for 8–10 h as in (a). Cell lysates were analyzed for luciferase activity. Bars represent the mean of triplicate samples. The data shown (a,b) are representative of three independent experiments. (c) Aliquots of cell lysates in (b) were analyzed with anti-HA or anti-Ras antibody.

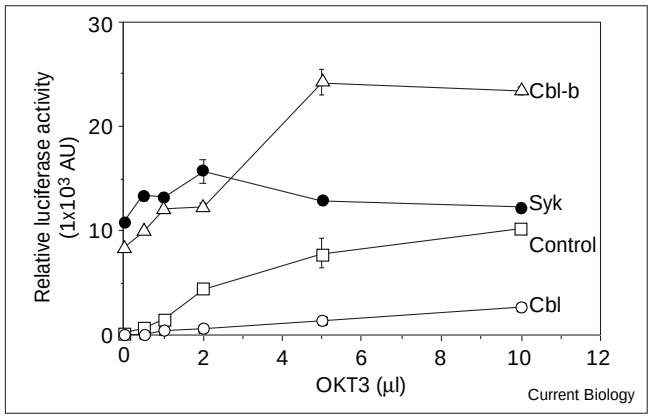
further demonstrated that this effect was mediated by the direct interaction between the Cbl-b PTB domain and the Tyr292 residue of Zap-70, as a loss-of-function mutation (G298E) in Cbl-b disrupted its interaction with Zap-70 and abolished Cbl-b-induced NFAT activation. Our results clearly indicate that Cbl-b plays a positive role in T-cell signaling, which leads to NFAT activation.

Two recent studies indicate that the Tyr292 residue of Zap-70 is a negative regulatory site: a Y292F Zap-70 mutant exhibits constitutive activation towards NFAT activation [S6,S7]. We recently observed the same result with a corresponding Syk Y316F mutant (our unpublished observations). Taken together, these results seem to agree with a hypothesis that these sites interact with a negative regulatory molecule(s). Thus, our data on Cbl-b as shown in the present study disagree with this model regarding the regulation of Syk/Zap-70. It is also shown, however, that Tyr292 is located in the linker region encompassing a negative regulatory domain, which contains binding sites for critically positive signaling molecules such as Vav [S8]. Removal of this linker region results in a Zap-70 mutant that is constitutively active [S7]. Thus, it is possible that the Y292F mutation of Zap-70 (or Y316F of Syk) may acquire a gain-of-function property via a structural change of this linker region, in addition to the disruption of its interaction with Cbl or Cbl-b. Clearly, future studies are needed to dissect the exact role of these binding sites for Cbl-family proteins in Zap-70 and Syk.

Our hypothesis that binding to Tyr292 of Zap-70 transduces either a positive and a negative signal is supported by the observation that Cbl-b(N), while still retaining the ability to bind Zap-70, does not induce constitutive activation of NFAT. Rather, it functions as a dominant-negative regulator of Cbl-b-induced NFAT activation. In addition, this inhibitory effect of Cbl-b(N) is markedly alleviated by the G298E mutation, suggesting that a direct interaction with Zap-70 accounts for the inhibitory function of Cbl-b(N). Consistent with this observation is our previous work on a Cbl-b fragment corresponding to Cbl-b(N), v-Cbl, which also interacts with Zap-70 [S9], but is unable to induce NFAT activation under either resting or ionomycin-stimulated conditions [S10].

The constitutive activation of NFAT by Cbl-b is reminiscent of an oncogenic Cbl mutant (70Z), which also causes constitutive NFAT activation [S10]. Both proteins require a Ca^{2+} signal and functional Ras to exert their effects, implying that their targets of action are upstream of these two signals. Indeed, we recently demonstrated that a similar loss-of-function mutation in 70Z has the same effect as Cbl-b G298E on NFAT activation, suggesting these two molecules employ a common mechanism, that is, direct targeting of their upstream binding protein Zap-70. One obvious difference between these two molecules is that 70Z but not Cbl-b synergizes with ionomycin treatment [S10] (Figure 1). Another difference is that while Cbl-b enhances anti-CD3-induced NFAT activation, 70Z inhibits this event [S11]. Although the exact

Figure S3



Comparison of Cbl proteins with Syk in the induction of NFAT transactivation. The experiment was performed as in Figure 1b. Cells were also transfected with a plasmid containing Syk cDNA (5 μg) for comparison. As shown here, Syk did not synergize with the TCR-induced signal to induce NFAT transactivation.

mechanism underlying these different effects remains to be explored, differential complex formations by these two molecules may explain the observed functional differences. Nevertheless, these functional differences agree with our model that interaction of the same Tyr292 residue in Zap-70 by different Cbl-family proteins can result in differential Zap-70-mediated signaling.

The endogenous expression of Cbl-b in T cells and other hematopoietic cells and its rapid tyrosine phosphorylation upon stimulation of TCR and other cytokine receptors [S12,S13] strongly suggest that Cbl-b is involved in antigen- or cytokine-receptor-mediated signaling. In the present study, we demonstrated that anti-CD3 stimulation enhances Cbl-b-induced NFAT activation. Titration experiments using different amounts of either Cbl-b plasmid for transfection or different amounts of anti-CD3 antibody for stimulation showed that a synergy between Cbl-b and TCR ligation does exist in the induction of NFAT activation, suggesting a direct connection between Cbl-b and TCR-mediated signaling. The failure to see a strong synergy between these two signals in some other experiments could suggest that Cbl-b is not a limiting factor in this process. Otherwise, tyrosine phosphorylation of Cbl-b induced by TCR ligation recruits Crk-L [S12]. Recently, a Cbl–Crk-L–Rap1 signal has been proposed to be responsible for T-cell anergy [S14]. It can be speculated that under anti-CD3-stimulated conditions, Cbl-b can also transduce a negative signal(s). A functional balance between positive and negative signals will determine the effect of Cbl-b on NFAT activation under anti-CD3-stimulated conditions. Other critical signaling molecules in T-cell activation such as Syk (Figure S3) or LAT [S15] do not synergize with TCR ligation either.

The functional implication of Cbl family proteins in the regulation of receptor-mediated signaling is further complicated by several other studies showing that Cbl, a negative regulator, also functions as a positive regulator of intracellular signal transduction: Cbl was reported to positively regulate a Src-mediated signaling pathway leading to bone resorption [S16], IL-4-induced cell proliferation [S17] or integrin-mediated cell spreading or cell adhesion [S18,S19], in addition to its attributed role as a negative regulator. Similarly, it was reported that Cbl-b binds Vav and inhibits Vav-mediated activation of Jun N-terminal kinase [S20,S21], although we do not observe a Cbl-b–Vav interaction in Jurkat T cells even when both Cbl-b and Vav are overexpressed [S12]. At present, the exact mechanism for the dual function of Cbl and/or Cbl-b remains unclear. These findings suggest, however, that both Cbl and/or Cbl-b can function as positive or negative regulators, depending on the specific signaling pathway and/or the particular cellular context. Clearly, additional studies are needed to elucidate the role(s) of Cbl-b and/or Cbl in intracellular signaling pathways. The current findings provide a framework for future studies on the function of Cbl-family proteins in TCR-mediated signal transduction, which may be crucial for the understanding of these molecules in the regulation of both normal and aberrant T cell responses.

Supplementary materials and methods

Antibodies

Polyclonal rabbit anti-Cbl-b (N-19 and H-121) antibodies were from Santa Cruz Biotechnology. A polyclonal rabbit anti-Cbl-b antibody suitable for immunoblotting was from O. Rosnet. Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology. Anti-hemagglutinin (HA) monoclonal antibody (12CA5) was from Boehringer Mannheim. Anti-Zap-70 monoclonal antibody was from Transduction Lab. Anti-CD3ε monoclonal antibody, OKT3, was purified from ascities using a protein-G–Sepharose affinity column. Horseradish peroxidase-conjugated $F(ab')_2$ fragments of donkey anti-rabbit IgG or sheep anti-mouse IgG were from Amersham.

Plasmids

The Cbl-b cDNAs encoding full-length protein (Cbl-b; amino acids 1–982), its amino-terminal region (Cbl-b(N); amino acids 1–349), a loss-of-function mutation (G298E) of Cbl-b(N), with an HA epitope at the carboxy-terminal end in an elongation factor promoter-driven mammalian vector were described previously [S12,S22]. A G298E mutation in full-length Cbl-b was made by site-directed mutagenesis (QuickChange, Stratagene). The NFAT–luc reporter plasmid was kindly provided by G. Crabtree. Plasmids encoding wild-type human Zap-70 or β-galactosidase in pEFneo were constructed in this lab. A plasmid encoding human Zap-70 with a Y272F mutation was from A. Chan. A dominant-negative Ras construct (RasN17) was described previously [S10].

Cell culture, transfection, and stimulation

Simian virus 40 T antigen (TAg)-transfected human leukemic Jurkat T cells (Jurkat-TAg) were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum and antibiotics. For protein expression in Jurkat-TAg cells, cells were transfected with appropriate amounts of plasmids (usually 3–10 μg total) by electroporation as previously described [S22]. For phosphotyrosine studies, cells were resuspended (2×10^7/ml) in 0.5 ml medium, equilibrated at 37°C for 5 min, and activated with OKT3 (4 μg/ml) for 5 min. Stimulation was terminated by adding 0.5 ml 2× Nonidet P-40 (NP-40) lysis buffer (2%

NP-40, 40 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM EDTA, 10 mM NaPiP, 10 mM NaF, 4 mM Na_3VO_4, 20 μg/ml each aprotinin and leupeptin). Cells were lysed for 10 min at 4°C and insoluble materials were removed by centrifugation at 15,000 × *g* (4°C for 10 min). For luciferase assays, cells were washed, resuspended in RPMI 1640 medium containing 0.2% FCS and incubated for 4–6 h in 24-well plates. The cells were then left unstimulated or stimulated with either purified OKT3 (4 μg/ml) or OKT3 ascites (1:500), ionomycin (100 ng/ml), or PMA (50 ng/ml) for another 8–10 h.

Immunoprecipitation and immunoblotting

Lysates (~1×10^7 cells) were mixed with antibodies for 2 h, followed by addition of 40 μl protein A/G Plus-Sepharose beads (Santa Cruz Biotechnology) for an additional hour at 4°C. Immunoprecipitates were washed four times with 1× NP-40 lysis buffer, and boiled in 20 μl 2× Laemmli buffer. Samples were subjected to SDS/10% polyacrylamide gel electrophoresis (PAGE) analysis and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were immunoblotted with the indicated primary antibodies (1 μg/ml), followed by horseradish-peroxidase-conjugated secondary antibodies. Membranes were then washed and visualized with enhanced chemiluminescence (ECL) detection system (Amersham). When necessary, membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.7/100 mM 2-mercaptoethanol/2% SDS for 1 h at 70°C with constant agitation, washed, and then reprobed with other antibodies as indicated.

Luciferase assay

The luciferase assay to determine the activation of reporter genes was described previously [S10]. Luciferase activity was determined in triplicate and expressed as arbitrary units (AU). The standard deviation among triplicates was ≤10%, and each experiment was repeated at least three times. As a proper control for transfection, cells were also cotransfected with a pEF plasmid encoding β-galactosidase (3 μg per sample). A portion of the cell lysates were analyzed for the enzyme activity using o-nitrophenyl-β-D-galatopyranoside as a substrate. The relative luciferase activity was normalized to the galactosidase enzymatic activity.

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