Cbl-mediated Regulation of T Cell Receptor-induced AP1 Activation

IMPLICATIONS FOR ACTIVATION VIA THE Ras SIGNALING PATHWAY*

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The functional role of Cbl in regulating T cell receptor (TCR)-mediated signal transduction pathways is unknown. This study uses Cbl overexpression in conjunction with a Ras-sensitive AP1 reporter construct to examine its role in regulating TCR-mediated activation of the Ras pathway. Cbl overexpression in Jurkat T cells inhibited AP1 activity after TCR ligation. However, AP1 induction by 4β-phorbol 12-myristate 13-acetate, which up-regulates Ras activity in a protein kinase C-dependent, TCR/tyrosine kinase-independent manner, was not affected by Cbl overexpression. Cbl overexpression also did not affect AP1 induction by an activated Ras protein or a membrane-bound form of the guanine nucleotide exchange factor Sos. In addition, activation of the mitogen-activated protein kinase Erk2 was decreased by Cbl overexpression. Therefore, Cbl regulates events that are required for full TCR-mediated Ras activation, and data are presented to support a model whereby Cbl regulates events required for Ras activation via its association with Grb2.

Perturbation of the T cell receptor (TCR) by antigen in the context of the appropriate major histocompatibility complex molecule initiates a cascade of events that involves the phosphorylation of proteins by tyrosine kinases (1). Two members of the Src family of tyrosine kinases, Fyn and Lck, are constitutively associated with the TCR and/or the CD4/CD8 coreceptors. Fyn and Lck appear to be responsible for the phosphorylation of conserved immune receptor tyrosine-based activation motifs on the cytosolic tails of the TCR-associated ζ and CD3 γ, δ, and ε chains (2–4). Phosphorylation of immune receptor tyrosine-based activation motifs leads to the recruitment of the Syk family tyrosine kinase Zap-70. Full activation of Zap-70 appears to require its phosphorylation by Fyn or Lck (5), and because there are multiple immune receptor tyrosine-based activation motifs on the TCR/CD3 ζ complex, signal amplification may occur at this step (6, 7).

The targets of Zap-70 kinase largely remain unknown, but two downstream events that are critical for T cell activation are the activation of phospholipase Cγ1 and p21ras (Ras) (8–11). Activation of phospholipase Cγ1 results in inositol phospholipid hydrolysis and the generation of second messengers that control protein kinase C activation and Ca2+ mobilization (12). Ras regulates the activity of multiple downstream effector pathways, including the mitogen-activated protein kinases Erk1 and Erk2 (10, 13, 14). Activation of the phospholipase Cγ1 and Ras signaling pathways culminates in the induction of transcription factors that regulate lymphokine production, receptor expression, and cell proliferation. Although the events that link Zap-70 activation with these downstream events are unclear, there are several early prominently tyrosine-phosphorylated proteins observed in response to TCR/CD3 engagement that may be important for signal transduction. One of these proteins is the product of the proto-oncogene c-cbl (15, 16).

Cbl is a cytosolic protein found in all hematopoietic cell lineages and some non-hematopoietic tissues such as testis, lung, and brain. It is a major target of tyrosine phosphorylation after the engagement of multiple receptor types, including the antigen receptors for T and B cells; Fc receptors; receptors for EGF, colony-stimulating factor-1, and granulocyte-macrophage colony-stimulating factor; and the thrombopoietin and erythropoietin receptors, suggesting that Cbl is involved in signal transduction pathways triggered by these distinct receptors (15–23). Cbl has no known enzymatic activity, and its function is unknown. However, the presence of multiple sites within Cbl with the potential for binding to SH2 and SH3 domains and the finding that Cbl interacts with a wide variety of proteins involved in signal transduction suggest that it may function as a complex adapter molecule (24–32).

One protein with which Cbl interacts is the adapter Grb2, a protein consisting of two SH3 domains and one SH2 domain (25, 28, 29, 33). Grb2 has recently been implicated as a regulator of the Ras activation pathway after TCR engagement by virtue of its ability to mediate the translocation of the guanine nucleotide exchange factor Sos from the cytosol to the membrane, where it can interact with and activate Ras (34–36). In Caenorhabditis elegans, a Cbl homolog, Sli-1, acts as a negative regulator of the Ras homolog, Let60, possibly by regulating the activity of Sem5, a Grb2 homolog (37). In addition, a transforming mutant Cbl protein, 70Z/3, has been implicated in the activation of Ras-dependent signaling pathways that are involved in the induction of NF-AT, a transcriptional regulator involved in interleukin-2 (IL-2) gene expression (38). Ras activation in T lymphocytes controls the assembly of the AP1 complex, a transcription factor complex that binds to site(s) within the IL-2 gene enhancer (11). We have therefore evaluated whether Cbl can regulate Ras-dependent signaling in T lymphocytes by using Cbl overexpression in conjunction with an AP1 reporter gene construct. We have found that Cbl overexpression inhibited TCR-induced AP1 and Erk2 activation.

We further observed a T cell activation-induced exchange of Cbl for Sos on Grb2, suggesting one potential mechanism by which Cbl could regulate Ras activation in T lymphocytes.

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The abbreviations used are: TCR, T cell receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; IL-2, interleukin-2; PMA, 4β-phorbol 12-myristate 13-acetate; HA, hemagglutinin; GST, glutathione S-transferase; SEAP, secreted alkaline phosphatase.
Cbl Regulates TCR-induced AP1 Activation

EXPERIMENTAL PROCEDURES

Reagents—46-Phorbol 12-myristate 13-acetate (PMA) was from Calbiochem Corp. (Nottingham, United Kingdom). The anti-CD3 monoclonal antibody (OKT3) was a generous gift from Ortho Biotech, Inc. (Raritan, NJ). The rabbit anti-Cbl antibody (C-15) that recognizes amino acids 892–906, the rabbit anti-Grb2 antibody (C-23), and the anti-Erk2 antibody (C-14) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-active mitogen-activated protein kinase antibody was from Promega (Madison, WI). The mouse anti-Sos1 antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). The R5 anti-Cbl antibody, which is directed against the carboxy-terminal region of the Cbl protein (amino acids 143–450), was provided by Dr. W. Langdon (University of Western Australia) (39). The anti-hemagglutinin (HA) monoclonal antibody (12CA5) was used as ascites from the corresponding hybridoma. All restriction enzymes were from New England Biolabs Inc. (Beverly, MA) or Life Technologies, Inc.

Plasmids—The AP-1 reporter vector (AP1-SEAP) and the myristoylated Sos expression plasmid (MSoSe) were provided by Dr. G. Crabtree (Stanford University Medical School) (35). The Ras expression vector pMEXneo-H-Ras4612 was provided by Dr. J. Labbora (Center for Biologic Evaluation and Research, Food and Drug Administration) (40). The tagged Erk2 expression plasmid pDNAII HA-er2/2 (Erk2-HA) was provided by Dr. S. Gutkind (National Institutes of Health). The human Cbl expression plasmid pCI-Cbl was generated by excising the Cbl cDNA from pJZ-Cbl (39) with BamHI and cloning it into the BamHI site of pGEM-11Zf(+) (Promega). Orientation was confirmed by restriction digestion. Cbl cDNA was then excised from a pGEM-11 clone with the correct orientation using XhoI and NotI and cloned into the expression vector pCl (Promega) through the expression sites XhoI and NotI at three sites. The pCl-c/-Cbl expression construct was made in an identical manner except that the cDNA was excised from pJZ-Cbl (39).

Cells and Transfections—The Jurkat cell line was used, which was a high CD4-expressing line generated by Drs. T. Oravecz and M. Norcross (Center for Biologies Evaluation and Research, Food and Drug Administration). Jurkat cells were maintained and transfected by electroporation as described (41).

AP1-SEAP Assays—Jurkat cells (107) were transfected with 4 µg of AP1-SEAP and the indicated expression vectors. Multiple transfections were pooled and were kept for 24 h. The cells were then resuspended in fresh medium, divided into wells at ~3.3 × 106 cells/well, and either left unstimulated or activated with immobilized OKT3 (20 µg/ well) or 10 nM PMA for 12 h. All stimulations were done in duplicate.

Western Blotting—Cbl Regulates TCR-induced AP1 Activation

The IL-2 enhancer is composed of numerous regulatory elements that work in concert to regulate IL-2 production after T cell activation (11). One of these elements consists of a conventional AP1 site. After TCR engagement, the induction of AP1 activity is dependent on the activation of multiple effector pathways by Ras (13, 43–47). We therefore used the reporter construct AP1-SEAP, which has the secreted alkaline phosphatase gene under the control of an IL-2 minimal promoter with upstream AP1-binding sites, to assess the effects of Cbl overexpression on TCR-induced AP1 activation. Jurkat T cells were transfected with AP1-SEAP and increasing amounts of a Cbl expression plasmid (pCl-Cbl) or a control vector (pCl-neo) and allowed to rest for 24 h. Cbl overexpression at this point in time was verified by Western blot analysis (Fig. 1A). The cells were then activated with OKT3 or the phorbol ester PMA, which stimulates the Ras/mitogen-activated protein kinase pathway in a TCR/tyrsoine kinase-independent manner (48, 49). Overexpression of Cbl led to a dose-dependent decrease in AP1 activity after stimulation with OKT3 that reached a maximum at ~30–40% of the control response (Fig. 1B). Cbl overexpression led to a similar decrease in AP1 activity in cells stimulated with the pharmacologic activator pervanadate (Fig. 1C), which mimics the activation events generated by TCR ligation (50, 51). However, responses to PMA were unaffected by Cbl overexpression (Fig. 1B).

Cbl overexpression also had no effect on the ability of an activated form of Ras (H-RasL46) or an activating form of Sos (MSoSe) to stimulate AP1 activity in Jurkat cells (Fig. 1D). These data suggest that Cbl overexpression affects AP1 activation prior to or independent of the activation of Ras itself and without directly affecting the ability of Sos to mediate Ras activation.

The viral homolog of Cbl, v-Cbl, is a fusion between the group-specific antigen protein of the CAS NS-1 retrovirus and the first 360 amino acids of the Cbl protein (39). This fusion protein localizes predominantly to the nucleus and is capable of inducing the transformation of NIH 3T3 cells. Removal of the viral group-specific antigen protein resulted in a protein (designated v/-Cbl) that could be found in both the cytoplasm and nucleus, but retained the ability to transform cells (39). To assess the effect that v/-Cbl had on AP1 activation in T cells, an expression plasmid encoding the truncated protein (pCl-c/-Cbl) was cotransfected with AP1-SEAP into Jurkat cells. Transfection of pCl-c/-Cbl resulted in expression of the truncated protein (Fig. 2B), but had no effect on AP1 activation after stimulation with OKT3 (Fig. 2C) or pervanadate (data not shown). These data indicate that v/-Cbl does not affect TCR-induced AP1 activation. In addition, these data suggest that the inhibitory effect of Cbl overexpression on AP1 activation requires the proline-rich carboxyl-terminal region of Cbl. This region contains multiple potential SH3-binding sites, including a binding site for Grb2, as demonstrated by the ability of a GST fusion protein encompassing a portion of this region to interact with and precipitate Grb2 (Fig. 3A, lower panel) (33). During T cell activation, Grb2 is thought to mediate the translocation of Sos from the cytosol to the membrane, thus allowing Sos to interact with and activate Ras. Cbl and Sos bind preferentially

by a Molecular Dynamics PhosphorImager using ImageQuant software (Version 3.3).

GST Fusion Proteins—The plasmid pGEX-KG-Grb2-N-SH3, which encodes a GST fusion protein of the amino-terminal SH3 domain of Grb2, was provided by Dr. W. Wong (NIH, National Institutes of Health) (42). The GST fusion protein of the proline-rich region of Cbl (pGEX-Cbl-polyp) corresponds to amino acids 458–689 of Cbl and was provided by Dr. M. Shapiro (Center for Biologics Evaluation and Research, Food and Drug Administration). All other GST-Grb2 fusion proteins were purchased from Santa Cruz Biotechnology Inc.
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A. Jurkat cells (10^7) were transfected with 12 μg of the indicated plasmid, cultured for 24 h, lysed, and analyzed by Western blotting (WB) for Cbl expression. B and C, Jurkat cells (10^7) were transfected with 4 μg of AP1-SEAP and the indicated amount of pCI-Cbl plasmid. The vector pCI-neo was added as needed to make the final amount of DNA in each transfection equal to 14 μg. After 24 h in culture, transfected cells were left untreated or were stimulated with immobilized OKT3 (20 μg/well), pervanadate (Perv.: 0.1 mM vanadate and 0.3 mM H_2O_2), or PMA (10 nM) for an additional 24 h. Supernatants were collected and assayed for alkaline phosphatase activity as indicated under "Experimental Procedures." The means ± S.D. of triplicates from a representative experiment of 10 (B) or three (C) performed are shown. -Fold activation was calculated as the ratio of the SEAP activity of the stimulated sample over the activity of the matched untreated control. D, Jurkat cells were transfected with 4 μg of AP1-SEAP, 10 μg of either pMEXneo-H-Ras^Lys12 or MSosE, and the indicated amount of pCI-Cbl. Cells received a total of 24 μg of DNA, with differences being made up with the pCI-neo vector. Cells were cultured for 24 h, and the supernatants were assayed for SEAP activity. Shown is the mean ± S.D. of triplicates from a representative experiment of three performed.

FIG. 1. Cbl overexpression regulates TCR-induced AP1 activation.

A, Nuclear localization of Cbl and the truncated Cbl protein (c/v-Cbl) with critical structural regions. The regions of Cbl recognized by the R5 and C-15 anti-Cbl antibodies are indicated. aa., amino acids. B, Jurkat cells were transfected with 10 μg of the indicated expression plasmid and assayed for protein expression after 24 h with the R5 anti-Cbl antibody. WB, Western blot. C, Jurkat cells were transfected with increasing amounts of an expression plasmid for a truncated form of Cbl (pCI-c/v-Cbl) or full-length Cbl (pCI-Cbl) as indicated and 4 μg of the AP1-SEAP reporter construct. All transfections contained a total of 14 μg of DNA, with the difference being made up with the control vector (pCI-neo). Cells were either left untreated or were stimulated with OKT3. The mean ± S.D. of a representative experiment out of four is shown.

FIG. 2. The carboxyl-terminal region of Cbl is required for regulation of AP1 activation. A, shown are the structures of Cbl and the truncated Cbl protein (c/v-Cbl) with critical structural regions. The regions of Cbl recognized by the R5 and C-15 anti-Cbl antibodies are indicated. aa., amino acids. B, Jurkat cells were transfected with 10 μg of the indicated expression plasmid and assayed for protein expression after 24 h with the R5 anti-Cbl antibody. WB, Western blot. C, Jurkat cells were transfected with increasing amounts of an expression plasmid for a truncated form of Cbl (pCI-c/v-Cbl) or full-length Cbl (pCI-Cbl) as indicated and 4 μg of the AP1-SEAP reporter construct. All transfections contained a total of 14 μg of DNA, with the difference being made up with the control vector (pCI-neo). Cells were either left untreated or were stimulated with OKT3. The mean ± S.D. of a representative experiment out of four is shown.

and in a mutually exclusive manner to the amino-terminal SH3 domain of Grb2 (data not shown and Refs. 25, 28, 29, 52). We therefore reasoned that Cbl could regulate AP1 complex assembly by controlling Ras activation via the Sos/Grb2 interaction. For Cbl to regulate the association of Sos with Grb2, it would need to dissociate from Grb2 after activation, freeing a pool of Grb2 that could then interact with Sos. We and others (25, 28, 33) have analyzed the ability of Cbl to associate with Grb2 under different activation conditions. In unstimulated T cells, Cbl readily associates with the amino-terminal SH3 domain of Grb2, as can be shown by the ability of Cbl to react with a GST fusion protein consisting of this Grb2 domain (Fig. 3A, upper panel). Cbl associates very weakly with the carboxyl-terminal SH3 domain of Grb2 and not at all with the SH2 domain, even after activation through the TCR (Fig. 3A and data not shown). Activation of Jurkat cells with OKT3 or pervanadate for 2 min
leads to a substantial decrease in the amount of Cbl recovered with the full-length Grb2 fusion protein and the GST-Grb2 N-terminal SH3 domain fusion protein (Fig. 3, A and B). The dissociation of Cbl from Grb2 is due to modifications of Cbl itself (either a direct post-translational modification or an induced structural change), rather than to Grb2, as demonstrated by the ability of a GST fusion protein of a fragment of the proline-rich region of Cbl to precipitate equal amounts of Grb2 from unactivated and activated Jurkat cells (Fig. 3A, lower panel). An activation-dependent dissociation of Cbl from Grb2 was also observed when Cbl was coprecipitated with Grb2 by using a Grb2 antiserum, confirming that it is a physiologic consequence of T cell activation (Fig. 3C). Interestingly, in unstimulated Jurkat cells, little Sos could be coprecipitated with Grb2, although the amount of Sos that associated with Grb2 increased considerably after activation (Fig. 3C). The inducible association of Sos with Grb2 after TCR engagement is consistent with the data of Nel et al. (53) and Ravichandran et al. (54), who found it to persist for up to 30 min. Our data indicate that Cbl and Sos interact with an interchangeable pool of Grb2 and suggest that Cbl can regulate TCR-mediated, Sos-dependent activation of the Ras pathway in T lymphocytes by competing with Sos for binding to the amino-terminal SH3 domain of Grb2.

To establish more directly the effect of Cbl overexpression on Ras signaling during T cell activation, the degree of activation of the Ras effector Erk2 was examined. Erk2 is a serine/threonine mitogen-activated protein kinase that mediates AP1 activation by phosphorylating the transcription factor Elk-1/TCF, which controls c-fos transcription (55, 56). In T cells, Ras is both necessary and sufficient for Erk2 activation (46). To examine Erk2 activity in Cbl-overexpressing cells, Jurkat cells were cotransfected with an influenza HA-tagged Erk2 expression plasmid (Erk2-HA) and pCI-Cbl or with pCI-c/v-Cbl as a control. The degree of induced activation of the tagged Erk2 protein was determined by Western blot analysis of the phosphorylation-induced molecular mass shift of Erk2-HA immunoprecipitates. Pervanadate treatment consistently resulted in decreased Erk2-HA activation in cells overexpressing Cbl compared with cells expressing c/v-Cbl, where most of the Erk2-HA was activated after pervanadate treatment (Fig. 4). In three separate experiments in which Erk2-HA activation was assessed by immunoblotting with an antibody that recognizes the active form of mitogen-activated protein kinase, cells overexpressing Cbl had a 34% decrease in the amount of active Erk2-HA compared with the c/v-Cbl overexpressors (data not shown). In contrast to pervanadate treatment, Erk2-HA activation by PMA was unaffected or slightly increased in cells overexpressing Cbl. These data indicate that Cbl overexpression negatively affects receptor-proximal events required for Ras activation during T cell stimulation. The fact that the magnitude of the inhibition of Erk2 activation by Cbl overexpression was not as great as that seen for AP1 activation most likely reflects non-linearity and unequal sensitivity between the two assays. Furthermore, Cbl overexpression may inhibit other Ras-sensitive pathways (such as the c-Jun kinase pathway) that also contribute to AP1 activation.

**DISCUSSION**

The current findings that Cbl overexpression can inhibit AP1 and Erk2 activation suggest that Cbl regulates events that are critical for Ras signaling during T cell activation and provide the first evidence of a role for Cbl in the regulation of a specific signaling pathway in T cells. Since Cbl overexpression does not affect AP1 activation induced by PMA or constitutively activated Ras or Sos proteins, our observations indicate that Cbl acts upstream of Ras. These data and the fact that Cbl interacts with a number of critical TCR-associated signaling elements (such as Lyn, Lck, ZAP-70, and Grb2) (25, 27–29, 31–33) suggest that Cbl regulates receptor-proximal activation events.

The presence of multiple protein interaction sites in Cbl, together with the documented association of Cbl with critical signaling elements and the absence of an enzymatic activity associated with Cbl, suggests it acts as a "complex" adapter molecule. In this role, Cbl may act by preassembling an ordered complex of effector and adapter molecules prior to TCR activation and facilitating efficient signal transduction after receptor engagement. In assembling such a complex in unactivated (resting) cells, Cbl may also regulate the interaction of specific effectors (such as Sos) with this complex. Such a function is analogous to that of the "scaffold" proteins in yeast, which help form ordered protein modules that permit sequential activation
of multiple effector molecules (57). Overexpression of Cbl could therefore negatively affect T cell activation by disrupting the physiologic stoichiometry of the activation complexes. For example, an increase in the intracellular concentration of Cbl may reduce its ability to achieve an ordered clustering of proteins. In addition, overexpression may allow Cbl to compete more effectively for Grb2 binding, thereby directly inhibiting the ability of effectors (e.g. Sos) to associate with Grb2 during activation. Tyrosine-phosphorylated forms of Cbl also associate with the SH2 domains of the adapters Crk (which binds the guanine nucleotide exchange factors Sos and C3G) and Nck (which binds Sos) (25, 26, 30, 58, 59). Both Crk and Nck have been implicated in Ras activation (26, 25, 58, 60). Therefore, Cbl may influence the association of these adapters with other proteins as well.

Our observation of an activation-induced exchange of Cbl for Sos on Grb2 suggests that Cbl may physiologically function by inhibiting the association of Sos with Grb2 in unactivated T cells. The mechanism responsible for the dissociation of Cbl from Grb2 after T cell stimulation is unknown, although a post-translational modification of Cbl itself is likely. Cbl is rapidly phosphorylated upon TCR engagement, reaching a maximum within ~2 min. Because the early time course of Cbl phosphorylation parallels that of the dissociation of Cbl from Grb2, it is likely that Cbl phosphorylation plays a role in regulating its association with Grb2. However, the recent finding that Cbl remains dissociated from Grb2 30 min after T cell activation, even though it is dephosphorylated at this point in time, suggests that factors in addition to tyrosine phosphorylation may contribute to the regulated interaction of Cbl with Grb2. These factors include the activation-induced ubiquitination of Cbl (22), serine/threonine phosphorylation of Cbl, and/or conformational changes induced by the association of Cbl with other proteins.

The recent work of Ota and Samelson (61), which demonstrates that Cbl overexpression in mast cells has a negative impact on Syk phosphorylation and function, led to the suggestion that Cbl may be able to directly modulate the activity of the kinases with which it associates. Although clearly Cbl needs to associate with Syk to regulate its activity, it still remains to be established whether this effect is due to Cbl directly modulating the activity of Syk or whether it is mediated by a sequestration of Syk by Cbl overexpression. Because Cbl has the potential to associate with ZAP-70 and phospholipase Cγ1, it will be important to determine how Cbl regulates the function of the kinases with which it associates and whether Cbl regulates the activation of these enzymes as well (15, 31, 32, 59).

Our data are consistent with and further extend the recent report by Lui et al. (38), which demonstrates that ectopic expression of the transforming 70Z/3 mutant Cbl protein in Jurkat cells can augment NF-AT activity to a small extent in unactivated Jurkat cells and very significantly in cells stimulated with PMA in the presence of ionomycin. The ability of 70Z/3 to mediate this effect was shown to be inhibited by a dominant-negative form of Ras. The NF-AT transcriptional element requires dephosphorylation of the NF-AT protein by the Ca2+-sensitive phosphatase calcineurin as well as activation of AP1 through the Ras signaling pathway. Therefore, these findings suggest that ectopic expression of 70Z/3 leads to increased AP1 activation, which can then synergize with ionomycin-induced calcineurin activity to mediate up-regulation of an NF-AT element. Although, Lui et al. were unable to show direct 70Z/3-mediated augmentation of an AP1-sensitive reporter construct, we have found that ectopic expression of the 70Z/3 mutant does augment the activity of the AP1-SEAP reporter used in our studies in both unactivated and OKT3-stimulated Jurkat cells.2 Differences in the promoter regions or the reporter protein (luciferase versus alkaline phosphatase) utilized by the two AP1 reporter constructs used in these studies probably account for the differences observed between our two laboratories in the ability of 70Z/3 to mediate AP1 activation. The mechanism by which 70Z/3 facilitates Ras activation is unknown, although the fact that it is constitutively phosphorylated suggests that it may behave as a constitutively active form of Cbl, consistent with its transforming capabilities. Lui et al. did not detect any effect of wild-type Cbl overexpression on PMA/ionomycin-mediated NF-AT activation. This is consistent with our observations that Cbl regulates activation signals proximal to the TCR and has no effect on the stimulation of downstream effectors such as protein kinase C or calcium by PMA or ionomycin, respectively.2

The finding that Cbl overexpression leads to a disruption of TCR-mediated signaling events is also consistent with recent reports of the effect of Cbl overexpression on EGF receptor (EGFR) signaling (62, 63). These reports demonstrated that overexpression of Cbl in fibroblasts leads to a decrease in EGFR phosphorylation and in the amount of Shc that becomes associated with the EGFR after EGF stimulation. The study by Ueno et al. (62) also suggests that Cbl can regulate EGF-mediated activation of the JAK/STAT signaling pathway. However, neither study (62, 63) was able to detect any effect on EGF-mediated activation of the Ras pathway. This apparent discrepancy with our results is most probably explained by the fact that the role of Cbl in specific signaling pathways may be cell type- and receptor-specific. For example, the relative amount of Cbl and Sos in different cell types would influence whether or not Cbl could compete with Sos for binding to Grb2. This may be the case in fibroblasts since Bowtell and Langdon (63) found only a marginal association of Cbl with Grb2 in 3T3 cells. This suggests that Cbl has a different stoichiometry of association with Grb2 in fibroblasts compared with T cells either because of its expression level relative to that of Sos or because of a cell-specific mechanism that regulates the ability of Cbl to bind to Grb2 in fibroblasts. Ueno et al. (62) also speculated that EGF-induced activation of the Ras pathway may proceed by an EGFR kinase-independent mechanism because the kinase activity of the ErbB2 receptor can compensate for that of the EGFR (62). Whether Ras activation via the ErbB2 receptor is affected by Cbl overexpression remains to be determined.

Although a preponderance of data suggests that Cbl acts as a complex adapter molecule in all cell types, little is known concerning the specific function of Cbl in hematopoietic cells. In addition, data increasingly suggest that the signaling pathways Cbl regulates are cell type-specific and that its mechanism of action is dependent on the molecular stoichiometry of expression of effector (and adapter) molecules in different cell types. By localizing the site of Cbl action in T cells to a point proximal to the TCR and upstream of Ras, our present findings form a basis for further investigations of the function of Cbl in cellular activation in the hematopoietic cell lineage.

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2 B. L. Rellahan and E. Bonvini, unpublished observations.
