Dual Regulation of T Cell Receptor-mediated Signaling by Oncogenic Cbl Mutant 70Z*

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

From the Division of Cell Biology, La Jolla Institute for Allergy and Immunology, San Diego, California 92121

We previously showed that an oncogenic Cbl mutant (70Z) is constitutively active in transcriptional activation of nuclear factor at activated T cells (NFAT). However, the mechanism underlying this effect remains unclear. Here we analyzed the effects of 70Z mutations at an amino-terminal loss of function site (Gly-306) and at carboxyl-terminal potential tyrosine or serine phosphorylation sites on association with signaling proteins and on NFAT activation. Mutation at Gly-306 of 70Z disrupted its association with Zap-70 and almost completely abolished its ability to induce NFAT activation under basal and ionomycin-stimulated conditions. However, mutations at potential tyrosine or serine phosphorylation sites had little effect. In fact, expression of 70Z with Tyr-700, Tyr-731, or Tyr-774 mutated to Phe increased NFAT activity in comparison with unmutated 70Z. These findings suggest that an amino terminus-mediated interaction of 70Z with Zap-70 plays a positive role and that a carboxyl terminus-mediated, phosphotyrosine-dependent interaction with their binding proteins plays a negative role in 70Z-mediated NFAT activation. In support of this notion are the observations that 70Z reduced T cell receptor-induced NFAT activation and that wild-type Cbl further inhibited this event, suggesting that both 70Z and wild-type Cbl employ a similar mechanism by which Cbl proteins dually regulate T cell receptor-mediated signaling.

‡ To whom correspondence should be addressed: Division of Cell Biology, La Jolla Institute for Allergy and Immunology, 10355 Science Center Dr., San Diego, CA 92121. Tel.: 619-678-4604; Fax: 619-558-3505; E-mail: yuncail@liai.org.

* This work was supported by Grant CA35299 from the United States Department of Health and Human Services (to A.A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The c-cbl (Casitas B-lineage lymphoma) proto-oncogene was originally isolated as a cellular homologue of v-cbl, a part of the transforming gene of the Cas NS-1 murine leukemia retrovirus (3, 4). The 120-kDa product of c-cbl, Cbl, consists of an amino-terminal v-Cbl homologous region, a Ring finger, and a carboxyl-terminal proline-rich domain containing several potential tyrosine phosphorylation sites. Cbl is rapidly phosphorylated on tyrosine residues in response to stimulation of various cell surface receptors, including the TCR (reviewed in Ref. 5), suggesting a critical role for Cbl in signal transduction pathways. The importance of Cbl in intracellular signal transduction is further emphasized by the observation that it associates with several other signaling molecules: e.g. the constitutive binding to Grb2 (6–9); phosphotyrosine (Tyr(P))-dependent interaction with phosphatidylinositol 3-kinase; PTB, phosphotyrosine binding; NFAT, nuclear factor at activated T cells (NFAT). How-
nal potential Tyr(P) or phosphoserine sites and analyzed their interactions with respective binding partners and effects on NFAT activation. We demonstrated that expression of the G306E mutant, which disrupted the interaction with Zap-70, abolished 70Z-induced NFAT activation under both basal and ionomycin-stimulated conditions. However, mutations at Tyr-700, Tyr-731, and Tyr-774, but not at other single tyrosine or serine residues, enhanced the basal and ionomycin-treated activation of NFAT. Our results suggest that the interaction of the amino-terminal PTB domain of 70Z with Zap-70 plays a positive role and that the interactions of its carboxy-terminal Tyr(P) residues with potential binding partners such as PI3-K and Crk-L can play a negative role in this event.

MATERIALS AND METHODS

**Antibodies**—Polyclonal rabbit anti-Cbl (c-15), anti-Crk-L, anti-TCR γ chain, and anti-Zap-70 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Tyr(P) monoclonal antibody 4G10, anti-Vav monoclonal antibody, and anti-p85 subunit of PI3-K polyclonal antibody were from Upstate Biotechnology (Lake Placid, NY). Anti-hemagglutinin (HA) monoclonal antibody, and anti-14-3-3 monoclonal antibody, were from Amersham. Anti-Tyr(P) monoclonal antibody was described previously (22, 23). Dual signal transduction was induced with 10 ng/ml OKT3 or ionomycin and 50 ng/ml phorbol myristate acetate (Sigma) for 8–10 h. Samples were subjected to SDS-10% polyacrylamide gel electrophoresis analysis and electrotransferred onto polyvinylidene difluoride membranes (Millipore). Membranes were immunoblotted with the indicated primary antibodies (usually 1 μg/ml), followed by horseradish peroxidase-conjugated secondary antibodies. Membranes were washed and visualized with an ECL detection system (Amersham). When necessary, membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 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 (38). 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.

RESULTS

**Expression of Y700F, Y731F, Y774F, or Y700/774F Enhanced 70Z-mediated NFAT Activation**—We previously showed that 70Z induces the transactivation of NFAT in synergy with a Ca2+ signal (38). However, the mechanism underlying this effect is unknown. To understand this mechanism, we first made several Tyr to Phe mutations at Tyr-700, Tyr-731, Tyr-774, or Tyr-700/774, which are shown to be the binding sites for Vav, PI3-K, and Crk-L (both Tyr-700 and Tyr-774), respectively (16, 18, 21, 38), and confirmed their respective roles for protein-protein interaction. As shown in Fig. 2A, the Y700F or Y774F single mutation had only a partial effect on Crk-L interaction. However, a Y700/774F double mutation completely abolished its interaction with Crk-L, indicating that both tyrosine residues are required for the Crk-L interaction (18). A Y731F mutation disrupted the interaction with p85, consistent with our previous observation (38). Under the same conditions, we did not detect Vav/70Z interaction as reported previously for Vav/Cbl interaction (21), suggesting that a Vav/70Z interaction is relatively weaker than that of 70Z with Crk-L or PI3-K.

Next we examined whether these mutants affect 70Z-mediated NFAT transactivation. Plasmids containing 70Z or its mutants were cotransfected into Jurkat-TAg cells with the pEF-neo reporter gene (38). Cells were left unstimulated or stimulated with OKT3 or ionomycin and assayed for luciferase activity. As shown in Fig. 2B, 70Z induced NFAT activation under resting conditions, which synergized with ionomycin and visualized with an ECL detection system (Amersham). Nonidet P-40 lysis buffer and boiled in 30 mgl/ml), followed by horseradish peroxidase-conjugated fragments of donkey anti-rabbit IgG or sheep anti-mouse IgG were from Amersham.

**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 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics. For protein expression in Jurkat-TAg T cells, cells were transfected with the appropriate amount of plasmids (usually 5–10 μg each) by electroporation as described previously (23). Cells were resuspended (2 × 107 cells/ml) in 0.5 ml of 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 of 2× Nonidet P-40 lysis buffer (2% Nonidet P-40, 40 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 4 mM Na3VO4, and 20 μg/ml each of aprotinin and leupeptin). Cells were lysed for 10 min at 4 °C and insoluble materials were removed by centrifugation at 15,000 g for 4 °C for 10 min. For luciferase assays, cells were washed, resuspended in RPMI 1640 medium containing 0.2% fetal calf serum, and incubated for 4–6 h in 24-well plates. The cells were then left unstimulated or stimulated with either OKT3 ascites (1:500) or purified OKT3 (3 μg/ml), ionomycin (100 ng/ml), or both ionomycin and phorbol myristate acetate (50 ng/ml) for another 8–10 h.

**Immunoprecipitation and Immunoblotting**—Lysates (1 × 107 cells) were mixed with antibodies for 2 h, followed by the addition of 40 μl of protein A/G Plus-Sepharose beads (Santa Cruz Biotechnology) for an additional hour at 4 °C. Immunoprecipitates were washed four times with 1× Nonidet P-40 lysis buffer and boiled in 30 μl of 2× Laemmli’s buffer. Samples were subjected to SDS-10% polyacrylamide gel electrophoresis analysis and electrotransferred onto polyvinylidene difluoride membranes (Millipore). Membranes were immunoblotted with the indicated primary antibodies (usually 1 μg/ml), followed by horseradish peroxidase-conjugated secondary antibodies. Membranes were washed and visualized with an ECL detection system (Amersham). When necessary, membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 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 (38). 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.
negative role in the TCR-mediated NFAT activation. This suggestion is supported by the observation from the point-mutated 70Z proteins. Increases in the NFAT-driven luciferase activity, albeit to slightly different degrees, were observed in cells transfected with Y700F, Y731F, Y774F, or Y700/774F under unstimulated conditions or OKT3- or ionomycin-stimulated conditions as compared with unmutated 70Z. The increases in NFAT activation were more obvious in ionomycin-treated samples. These results suggest that these tyrosine residues are not responsible for the observed ability of 70Z in NFAT transactivation. Rather, interactions of 70Z with its binding partners such as PI3-K or Crk-L via these residues may play a negative role in this event.

Although the tyrosine residues at 700, 731, and 774 are known to be responsible for the protein-protein interactions, as also shown above, there are other potential tyrosine residues whose nature of tyrosine phosphorylation or potential role in protein-protein interactions are unclear. Therefore, we made additional point mutations at Tyr-552, Tyr-674, and Tyr-735, a single point mutation at Tyr-674F mutant did not express well and was not included in these experiments. These mutants were then analyzed for their ability to induce NFAT transactivation. As shown in Fig. 2D, Y552F, Y735F, and Y869/831F showed only slightly enhancing effects on NFAT transactivation as compared with 70Z in unstimulated conditions or OKT3- or ionomycin-stimulated conditions. OKT3. However, Y8F, a mutant with all eight carboxy-terminal tyrosine residues mutated, markedly enhanced its ability to activate NFAT under unstimulated or stimulated conditions. This result suggests that tyrosine residues at 552, 735, 869, and 871 are not critical for 70Z-mediated NFAT transactivation. The observed effect of Y8F may represent a synergy of the Y700F, Y731F, and Y774F mutations.

Effects of 70Z Mutants Deficient in 14-3-3 Binding on NFAT Activation—We previously demonstrated that Cbl interacts with 14-3-3 in an activation-dependent manner via two phosphoserine-containing motifs in Cbl (22, 23). Of these motifs, Ser-619 and Ser-639 have been predicted to be the serine phosphorylation sites responsible for the binding (23, 43). We then constructed a S619A single mutant and a S619/639A double mutant in 70Z and analyzed their interaction with 14-3-3. As shown in Fig. 3A, a mutation at Ser-619 reduced both basal and stimulation-induced interaction with 14-3-3; mutation at both the Ser-619 and Ser-639 sites abolished stimulation-induced 14-3-3 binding, although a residual basal level of 70Z/14-3-3 association remained. Next, we examined the effects of these two mutants on NFAT activation. Mutations at either single site (S619A) or double sites (S619/639A) showed only a slightly higher activation of NFAT as compared with 70Z under either unstimulated conditions or OKT3- or ionomycin-stimulated conditions (Fig. 3B). Analysis of cell lysates with anti-HA revealed comparable amounts of proteins among all

Effects of 70Z Tyr to Phe mutants on NFAT activation. A, Jurkat-TAg T cells were transfected with plasmids containing HA-tagged 70Z or its mutants including Y700F, Y731F, Y774F, or Y700/774F (3 μg of each). Thirty-six h later, lysates from 1 × 10⁶ unstimulated (−) or OKT3-stimulated (+) T cells were immunoprecipitated with anti-HA. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and immunoblotted with anti-p85 or anti-Crk-L as indicated. The membrane was reprobed sequentially with anti-Tyr(P) and anti-HA. Molecular weight markers are indicated on the gel. B, Jurkat-TAg cells were transfected with empty vector or the indicated 70Z plasmids (3 μg of each) plus 3 μg of NFAT-luc reporter plasmid. Twenty-four h later, cells were left unstimulated (Control), or stimulated with OKT3 ascites (1:500) or ionomycin (Iono; 100 ng/ml) for 6–10 h. Cells were then lysed, and the luciferase activity was determined. Bars represent the mean of triplicate samples. Standard deviations are depicted by the error bars. B represents five separate experiments. C, Jurkat-TAg cells were transfected with plasmids encoding HA-tagged 70Z or its mutants as indicated. Cells were analyzed as described in A. D, effects of 70Z mutants on NFAT activation. The experiment was performed as described in B. Bars represent the mean ± S.D. of triplicate samples. D represents four separate experiments.

FIG. 2. Effects of 70Z Tyr to Phe mutants on NFAT activation.
the samples (Fig. 3C). Although 14-3-3 may still have some effects on 70Z in these mutants because of remaining basal level interaction, this result suggests that an OKT3-stimulated, enhanced 70Z/14-3-3 interaction is not critical in 70Z-induced NFAT activation.

A Loss of Function G306E Mutant Was Critical for 70Z-mediated NFAT Activation—Previous studies have shown that a point mutation (G306E) in v-Cbl, which corresponds to a loss of function mutation in Sli-1, a Caenorhabditis elegans Cbl homologue, disrupts its interaction with PTKs including Zap-70 (28) and ablates v-Cbl-induced cell transformation (37, 44). Subsequent studies, including our own, demonstrated that the Zap-70 (28) and ablates v-Cbl-induced cell transformation (37, 44). Subsequent studies, including our own, demonstrated that

Syk/Zap-70. In addition, the increased inhibition of NFAT activity by G306E under OKT3 stimulation could be explained by a Tyr(P)-mediated negative signaling, in agreement with the aforementioned observations (Fig. 2).

The G306E Mutant Showed Reduced Tyrosine Phosphorylation and Disrupted the 70Z/Zap-70 Interaction—We have previously shown that the interaction of the Cbl PTB domain with the Syk Tyr-316 residue is required for maximal tyrosine phosphorylation of Cbl (30). To further explore the mechanism underlying the G306E-mediated effect on NFAT activation, we first examined whether the G306E mutation affects its tyrosine phosphorylation. Lysates prepared from cells transfected with empty vector, HA-tagged 70Z, or G306E left unstimulated or stimulated with OKT3 were immunoblotted with anti-Tyr(P). As compared with 70Z, G306E showed a markedly reduced tyrosine phosphorylation (Fig. 5A, top panel). Reprobing the membrane with anti-HA indicated that both 70Z and G306E were expressed at a similar level (bottom panel).

We then examined whether the reduced tyrosine phosphorylation of G306E resulted from a disruption of its interaction with Zap-70. The same cell lysates were then immunoprecipitated with anti-HA and immunoblotted with anti-Tyr(P). As shown in Fig. 5B, top panel, a ~70-kDa Tyr(P)-containing protein was coimmunoprecipitated with anti-HA from cells overexpressing HA-tagged 70Z under OKT3-stimulated conditions, which comigrated with Zap-70. Reprobing the same membrane with anti-Zap-70 failed to reveal Zap-70, suggesting that the nature of the 70Z/Zap-70 interaction is relatively weak. This weak interaction was consistent with the observations reported by other groups (24, 29). Importantly, the same Tyr(P)-containing protein, most likely Zap-70, was not detected or was only weakly detected in anti-HA immunoprecipitates from cells overexpressing HA-tagged G306E under the same conditions. This result suggests that an evolutionarily conserved loss of function mutation in 70Z disrupts its interaction with Zap-70, which is required for the maximal tyrosine phosphorylation of 70Z.

Next we examined whether the G306E mutation has any effect on tyrosine phosphorylation and its interaction with Zap-70 under ionomycin-stimulated conditions. To this end, we coexpressed Zap-70 with 70Z or G306E in Jurkat-TAg cells. As shown in Fig. 5C, coexpression of Zap-70 with 70Z induced the
tyrosine phosphorylation of 70Z under resting or ionomycin-stimulated conditions. Ionomycin stimulation caused only a slight increase in the tyrosine phosphorylation of 70Z. However, the tyrosine phosphorylation of G306E was not detectable under the same conditions. We then analyzed the interaction of 70Z or G306E with Zap-70 under unstimulated or ionomycin-stimulated conditions. Jurkat-TAg cells were cotransfected with Zap-70 plus 70Z or G306E. Lysates prepared from unstimulated cells (-) or cells stimulated with ionomycin (100 ng/ml; 5 min) were analyzed with anti-Tyr(P) and then reprobed with anti-HA. The position of 70Z was indicated. Lysates were immunoprecipitated with anti-HA, immunoblotted with anti-Tyr(P), and then reprobed with anti-HA. The position of 70Z or 70Z was indicated. D, effect of overexpression of 70Z or G306E on the tyrosine phosphorylation of Zap-70. Jurkat-TAg cells transfected with empty vector or with plasmids containing 70Z or G306E (3 μg of each) were left unstimulated (-) or stimulated with OKT3 (+). Cell lysates were immunoprecipitated with anti-Zap-70 and immunoblotted with anti-Tyr(P). The position of Zap-70 (top panel) or phospho-ζ (middle panel) is indicated by arrows. The same membrane was reprobed with anti-Zap-70 (bottom panel).

Previous studies have shown that 70Z interacts with and enhances the tyrosine phosphorylation and the kinase activity of the EGFR and PDGF receptors, which is proposed to be responsible for 70Z-induced cell transformation in fibroblasts (36, 37). To examine whether 70Z-induced NFAT transactivation is mediated by a similar mechanism (namely, the activation of Zap-70), we compared the tyrosine phosphorylation of Zap-70 among cells transfected with empty vector, 70Z, or G306E by analyzing anti-Zap-70 immunoprecipitates with anti-Tyr(P). As shown in Fig. 5D, top panel, almost no difference was observed among all the samples under OKT3-stimulated conditions. This result suggests that 70Z does not significantly affect the tyrosine phosphorylation and probably the kinase activity of Zap-70. In agreement with this suggestion is the observation that overexpression of 70Z or G306E did not change the amount of the tyrosine phosphorylated TCR ζ chain coimmunoprecipitated by anti-Zap-70 (Fig. 5D, middle panel), suggesting that 70Z did not affect the ability of Zap-70 to bind to TCR. Equivalent amounts of Zap-70 were detected in all the samples (Fig. 5D, bottom panel).

**Functional Role of Wild-Type Cbl in NFAT Transactivation**—Cbl has been demonstrated to be a negative regulator in several mammalian systems. Recently, it was shown that it is also a negative regulator in TCR-induced AP-1 activation (45). In principle, Cbl can also be a negative regulator in TCR-mediated NFAT activation because the NFAT promoter used in our studies consists of a NFAT binding site and an AP-1 binding site and requires both a Ca2+ signal and a Ras-dependent signal for its activation (39, 40). We have previously shown that wild-type Cbl has no effect on basal and ionomycin-stimulated NFAT activation (38). To examine whether wild-type Cbl has any effect on TCR-mediated NFAT activation, we transfected Jurkat-TAg cells with empty vector, 70Z, or wild-type Cbl plus NFAT-luc reporter plasmid and analyzed the NFAT-driven luciferase activity. As shown in Fig. 6, wild-type Cbl inhibited TCR-induced NFAT by 90%, which was stronger than 70Z-mediated inhibition under the same conditions. This inhibitory role of wild-type Cbl in TCR-induced NFAT transactivation is specific, because the overexpression of Cbl had no effect or only a subtle effect on ionomycin- (Fig. 6) or ionomycin plus phorbol myristate acetate-induced NFAT activation (data not shown). This result indicates that Cbl is a negative regulator in TCR-induced NFAT activation.

**DISCUSSION**

We previously showed that oncogenic Cbl mutant 70Z induces NFAT activation in T cells in synergy with a Ca2+ signal and in a functional Ras-dependent manner (38). Dissecting 70Z-mediated signaling using our established system could shed light on the biological function of Cbl proteins in T cell activation and leukemogenesis. Here we analyzed the biological role of an amino-terminal loss of function (Gly-306) site, the carboxyl-terminal potential Tyr(P), or phosphoserine sites of 70Z in the induction of NFAT, a critical component of interleukin-2 and other cytokine genes. We demonstrate that a mutation at Gly-306 abrogated 70Z-induced NFAT transactivation.
under both basal and ionomycin-treated conditions. However, mutations at Tyr-700, Tyr-731, Tyr-774, and Tyr-700/774, but not other Tyr to Phe or Ser to Ala mutations, increased both basal and ionomycin-treated NFAT activation. Our results suggest that 70Z plays both a positive and a negative role via its interaction with different targeting molecules.

In the present study, we clearly demonstrate that 70Z-mediated NFAT activation is mediated by its interaction with upstream PTKs, most likely Zap-70, because a loss of function mutation at Gly-306, which disrupts the interaction with Zap-70, almost completely abolished its ability to induce NFAT activation under both basal and ionomycin-stimulated conditions. This observation is consistent with the following previous findings: a corresponding mutation in Sli-1, a Cbl homologue in C. elegans, ablates Sli-1-mediated negative signaling (46); and a G306E mutant of v-Cbl disrupts its interaction with Zap-70 (28) or with EGF and PDGF receptors and abolishes its transforming ability in NIH3T3 cells (37, 44). Indeed, it was shown that by binding to EGF and PDGF receptors, 70Z enhances their tyrosine phosphorylation and kinase activity, which provides a mechanistic insight into 70Z-mediated cell transformation (36, 37). The observed NFAT activation by 70Z could use a similar mechanism, i.e. the activation of Zap-70 in T cells. However, 70Z does not have a significant effect on the tyrosine phosphorylation of Zap-70. Our result is consistent with the following observation in basophilic cells: cotransfection of 70Z overexpression of constitutively active PI3-K inhibited TCR-Crk-L. This notion is supported by the following findings: 1) an interaction with their binding partners such as PI3-K or Crk-L, not from structural changes from the Tyr to Phe mutation. However, it cannot be excluded that these tyrosine residues may bind other SH2-containing proteins, which are mediators for the observed OKT3-induced inhibition of NFAT by 70Z. Other possibilities such as the removal of 70Z from competing intracellular interactions, which may also enhance NFAT transactivation, cannot be ruled out either. In any case, it can be concluded that these tyrosine residues (Tyr-700, Tyr-731, or Tyr-774) play a negative role in 70Z-induced NFAT activation.

Taken together, our results clearly suggest a dual regulatory mechanism by which 70Z participates in TCR-mediated signaling: one by up-regulating the upstream PTKs (Syk/Zap-70) that it binds, and another by down-regulating downstream signaling via Tyr(P)-dependent interactions with its binding partners such as PI3-K and/or Crk-L (Fig. 7). The positive signal induced by 70Z/Zap-70 interaction is critical in ionomycin-stimulated NFAT activation. Mutation of the 70Z/Zap-70 interaction such as G306E disrupts this positive signal and thus abolishes ionomycin-induced NFAT activation. In the absence of a 70Z/Zap-70-induced positive signal, a Tyr(P)-dependent negative signal becomes dominant, leading to an enhanced inhibition of OKT3-mediated NFAT activation. This model may also have significant implications for the biological function of wild-type Cbl. In the case of wild-type Cbl, an amino terminus-mediated interaction of wild-type Cbl with PTKs contributes to a negative regulatory function of Cbl. This model is consistent with sev-

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2 Z. Zhang, C. Elly, A. Altmann, and Y.-C. Liu, unpublished data.
eral recent studies; for example, Cbl is a negative regulator of Syk (33) or a Cbl-mediated Crk-L-C3G-Rap1 signaling pathway is responsible for the defect in interleukin-2 production in anergic T cells (48). However, it is not known at present how the interaction of PTKs with the amino-terminal PTB domain of wild-type Cbl induces a negative one. Clearly, additional studies are needed to elucidate the molecular mechanism by which Cbl family proteins regulate the upstream Syk/Zap-70 family kinases with which they associate. These studies will be critical to understand the involvement of Cbl proteins in the regulation of TCR- or other cell surface receptor-mediated signaling pathways.

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