Differential effects of Cbl and 70Z/3 Cbl on T cell receptor-induced phospholipase Cγ-1 activity

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Abstract We demonstrate that the differential effects Cbl and oncogenic 70Z/3 Cbl have on Ca^{2+}/Ras-sensitive NF-AT reporters is partially due to their opposing ability to regulate phospholipase Cγ1 (PLCγ1) activation as demonstrated by analysis of the activation of an NF-AT reporter construct and PLCγ1-mediated inositol phospholipid (PI) hydrolysis. Cbl over-expression resulted in reduced T cell receptor-induced PI hydrolysis, in the absence of any effect on PLCγ1 tyrosine phosphorylation. In contrast, expression of 70Z/3 Cbl led to an increase in basal and OKT3-induced PLCγ1 phosphorylation and PI hydrolysis. These data indicate that Cbl and 70Z/3 Cbl differentially regulate PLCγ1 phosphorylation and activation. The implications of these data on the mechanism of Cbl-mediated signaling regulation are discussed.

Key words: Cbl; 70Z/3 Cbl; Phospholipase Cγ-1; Ca^{2+}; T cell receptor signaling; NF-AT

1. Introduction

Engagement of the T cell receptor (TCR) by antigen presented in the context of the major histocompatibility complex triggers multiple biochemical cascades that ultimately lead to T cell activation and proliferation. Two of the major pathways activated by TCR engagement are the Ras and phospholipase Cγ-1 (PLCγ1) pathways [1–4]. Although much is known concerning the signaling cascades following Ras and PLCγ1 activation, comparatively less is known about the link between TCR-induced tyrosine kinase activation and the activation of Ras and PLCγ1. Proteins that act as major substrates for tyrosine phosphorylation immediately following TCR engagement are likely to play an important role in signal transduction. One such protein is the adapter Cbl.

The *c-cbl* protooncogene was identified as the cellular homologue of *v-cbl*, the transforming gene of the Cas NS-1 retrovirus [5,6]. Another oncogenic form of Cbl, 70Z/3 Cbl, was isolated from a pre-B lymphoma and has an internal 17 amino acid deletion from amino acids 366–382 which results in a protein that is hyperphosphorylated and transforming in fibroblasts [7]. Cbl is a major substrate for tyrosine phosphorylation following stimulation via a number of different receptors including the T and B cell antigen-specific receptors [8–13] (for a complete review see [14]). Cbl is a complex protein with multiple, diverse, protein interaction domains [6,15–20]. These diverse domains have been shown to regulate the interaction of Cbl with important signaling molecules, such as Fyn, ZAP-70, Grb2, inositol phospholipid (PI)3K, Vav and PLCγ1 [9,10,15,16,18,21–24].

Forced over-expression of Cbl has been shown to inhibit signal transduction in various cellular systems. For example, Cbl over-expression in mast cells inhibits the function of the tyrosine kinase, Syk [25], while in fibroblasts it leads to inhibition of epidermal growth factor (EGF) receptor signaling [26,27]. We have demonstrated that Cbl over-expression inhibits TCR-induced activation of the Ras/MAPK pathway [28]. Furthermore, Sli-1, a Cbl homologue in *Caenorhabditis elegans*, was shown to act as a negative regulator of the Ras homologue, Let60, possibly by affecting the function of Sem5, a Grb2 homologue [29]. Taken together these studies suggest that Cbl is a negative regulator of signal transduction.

Several recent studies have utilized reporter constructs under the control of NF-AT elements to investigate the ability of wild type (WT) Cbl and the oncoprotein, 70Z/3 Cbl, to regulate signaling in T cells [30–32]. Optimal activation of *cis*-regulatory NF-AT sites requires both Ras-inducible AP-1 factors, as well as activation of NF-AT transcription factors by the calcium-sensitive phosphatase, calcineurin [33]. NF-AT reporters can therefore be used to examine activation of both the Ras and the Ca^{2+} signaling pathways. The latter pathway depends upon activation of PLCγ1 in T lymphocytes. Previous studies indicated that over-expression of WT Cbl inhibits receptor-mediated NF-AT activation, while ectopic expression of 70Z/3 Cbl leads to an increase in basal and ionomycin-stimulated NF-AT activity [30,32]. Since we have previously shown that Cbl over-expression inhibits TCR-induced Ras activation, it is possible that the inhibitory effect of Cbl over-expression on NF-AT reporter activity is solely due to inhibition of AP1 activation [28]. It was therefore of interest to confirm whether Cbl and 70Z/3 Cbl regulate NF-AT activation exclusively through their ability to regulate Ras. Contrary to our expectations, this report presents data that indicates that Cbl regulates TCR-induced PLCγ1 activation and that the opposing effects of WT Cbl and 70Z/3 Cbl on NF-AT activity are due, in part, to their differential ability to regulate PLCγ1 phosphorylation and activation. Therefore, Cbl has the ability to regulate events required for TCR-induced stimulation of both the PLCγ1/Ca^{2+} and Ras pathways.

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2. Materials and methods

2.1. Reagents

4β-Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem (Nottingham, UK). The anti-CD3 monoclonal antibody (OKT3) was from Ortho Biotech (Raritan, NJ, USA). The rabbit anti-Cbl antibody was purchased from Santa-Cruz Biotechnology (Santa Cruz, CA). The anti-phosphotyrosine antibody, 4G10, and the mixed monoclonal anti-PLCγl were purchased from Upstate Biotechnology (Lake Placid, NY). The anti-phosphotyrosine antibody, PY54, was purchased from Transduction Laboratories (Lexington, KY). The anti-hemagglutinin (HA) monoclonal antibody (12CA5) and the anti-TCR antibody, C305 were used as ascites from the corresponding hybridoma. The C305 hybridoma was a gift of Dr. Arthur Weiss (University of California, San Francisco, CA).

2.2. Plasmids

The pGreenLantern™-1 expression vector was from Life Technologies (Grand Island, NY). The NF-AT reporter vector (NF-AT-SEAP) was provided by Dr. Gerald Crabtree (Stanford University Medical School) [34]. Dr. Neil Clipstone (Northwestern University Medical School) provided the activated calcineurin expression plasmid (CNMUT2B). The Ras expression vector, pMEXneo-H-Ras^{Lys12}, was provided by Dr. Jorge Laborda (Center for Biological Evaluation and Research, Food and Drug Administration) [35]. The HA-tagged bovine PLCγl expression plasmid, pCIneo-PLCγl(HA), and the human Cbl expression plasmid, pCI-Cbl, were described previously [28,36]. The HA-tagged pJZEN/Neo 70Z/3 Cbl vector was a generous gift of Dr. Wallace Langdon (University of Western Australia). To generate non-HA tagged pCI-70Z/3 Cbl, the *Blp*I site of Cbl was used to re-clone the 70Z/3 mutation into the non-HA tagged pCI-Cbl vector.

2.3. Cells and transfections

The Jurkat cell line used was a high CD3 expressing line generated by Dr. Hana Golding (Center for Biological Evaluation and Research, Food and Drug Administration). Cells were transfected as previously described [37].

2.4. NF-AT-SEAP assays

Jurkat cells (10^7) were transfected with 7 μg of NF-AT-SEAP and the indicated expression vectors. Cells were stimulated and supernatants assayed for alkaline phosphatase activity as previously described [28].

2.5. Cell sorting, labeling and analysis of inositol phosphates

Cells transfected with pGreenLantern™-1 and the indicated expression vectors were rested for 24 h. Live cells were recovered from a Ficoll gradient and sorted on a Becton/Dickinson FACStar Plus. Inositol phosphate generation was measured as described [38]. For all transfection experiments, the data were internally normalized for the amount of total *myo*-[2-^{3}H]inositol incorporation and expressed as the percent change from untransfected cells. Data from three separate experiments were analyzed by ANOVA and differences within each group were compared by *t*-test.

2.6. Stimulation, immunoprecipitation

For TCR stimulation, cells were activated with either (1) the anti-TCR antibody, C305 (1:10 dilution of supernatant), or (2) the anti-CD3 antibody, OKT3, for 2 min at 10 μg/10^7 cells, or cells were coated with 10 μg/10^7 cells of OKT3 F(ab′)$_2$ for 15 min at 4°C, washed, and activated with 50 μg/10^7 cells of affinity-purified goat antibody to mouse IgG (Kirkegaard and Perry, Gaithersburg, MD) for 2 min. Post-nuclear lysates were immunoprecipitated with the indicated antibodies. For anti-phosphotyrosine immunoprecipitations, samples were boiled in 1% SDS, diluted to 0.1% SDS in 1×lysis buffer, immunoprecipitated, and proteins eluted with 100 mM phenyl-phosphate, prior to SDS–PAGE and Western blot analysis.

3. Results

3.1. Cbl over-expression inhibits TCR-induced NF-AT activation

Consistent with a previous observation [31], cells over-ex-

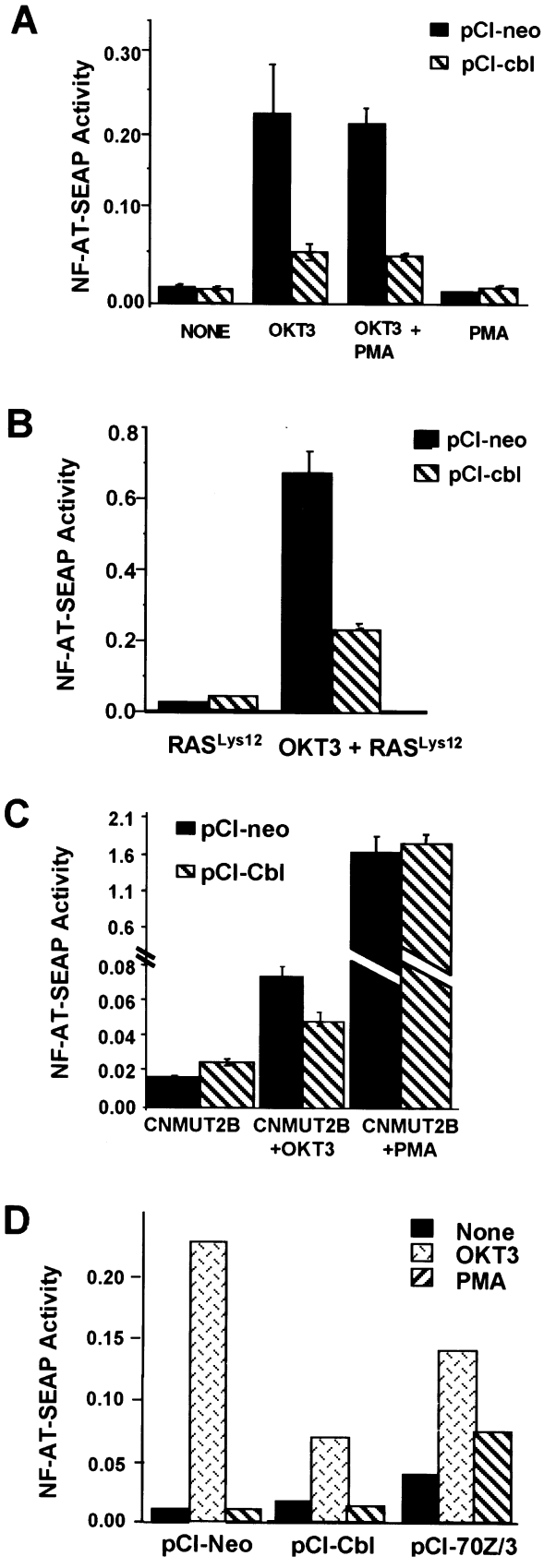

Fig. 1. Cbl over-expression regulates the PLCγ1/Ca^{2+} pathway upstream of Ca^{2+} mobilization. A: Jurkat cells were transfected with 7 μg of NF-AT-SEAP and 5 μg of either pCI-Cbl or pCI-neo. After 24 h in culture, transfected cells were left untreated or stimulated with immobilized OKT3 (20 μg/well), immobilized OKT3 plus PMA (10 nM), or PMA alone. Supernatants were collected and assayed for alkaline phosphatase activity. The mean ± S.D. of triplicate samples is shown. B: Jurkat cells were transfected with 7 μg of NF-AT-SEAP, 10 μg of pMEXneo-H-Ras^{Lys12} and 5 μg of either pCI-Cbl or pCI-neo. Cells were cultured for 24 h and either left untreated or stimulated with OKT3. Supernatants were assayed for SEAP activity. Shown is the mean ± S.D. of triplicates. C: Jurkat cells were transfected with 7 μg of NF-AT-SEAP, 0.5 μg of CNMUT2B and 5 μg of either pCI-Cbl or pCI-neo. After 24 h, the cells were either left untreated or stimulated with OKT3 or PMA as indicated. Supernatants were assayed for SEAP activity. Shown is the mean ± S.D. of triplicates. D: Cells were transfected with the indicated constructs as in A and treated with the indicated stimuli.
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pressing Cbl demonstrate approximately a 70% decrease in TCR-induced NF-AT activity compared to control cells (Fig. 1A). Because Cbl over-expression inhibits TCR-induced AP-1 activation [28] which is required for optimal activity of the NF-AT-SEAP reporter, the decrease in OKT3-induced NF-AT-SEAP activity in Cbl over-expressing cells could be due to inhibition of AP-1 activation. To overcome the inhibitory effect Cbl has on TCR-induced AP-1 activation, cells were activated either with a combination of OKT3 and the phorbol ester, PMA, or a combination of OKT3 and a constitutively active Ras protein, Ras^{Lys12}. Both PMA and Ras^{Lys12} stimulate AP-1 activity in a TCR-independent manner which is unaffected by Cbl over-expression [28]. Therefore, PMA and Ras^{Lys12} will bypass any inhibitory effect of Cbl over-expression on AP-1 activation, while TCR cross-linking with OKT3 will activate the PLCγ1/Ca^{2+} pathway with ensuing NF-AT transcriptional activation. Any reduction in NF-AT-SEAP activity observed under these experimental conditions will be due to an inhibition of TCR-induced activation of NF-AT transcription factors through the PLCγ1/Ca^{2+} pathway. As shown in Fig. 1A,B, Cbl over-expressing cells stimulated with OKT3 and PMA or OKT3 and Ras^{Lys12} maintain a 70% decrease in NF-AT-SEAP activation compared to control cells. These data indicate that Cbl over-expression can inhibit activation of NF-AT transcription factors.

Because the translocation of NF-AT transcription factors to the nucleus is controlled by calcineurin activity, we next determined if Cbl over-expression acts upstream or downstream of calcineurin. To this end we utilized an activated calcineurin construct (CNMUT2B) that leads to the constitutive dephosphorylation and nuclear translocation of NF-AT independent of TCR signaling. Jurkat cells were transiently transfected with NF-AT-SEAP together with CNMUT2B and either pCI-neo or pCI-Cbl. Because optimal activation of the NF-AT-SEAP reporter still requires AP-1 activation even in the presence of activated calcineurin, activation of AP-1 was accomplished by stimulation with OKT3 or PMA. PMA was used as a TCR-independent and Cbl-insensitive

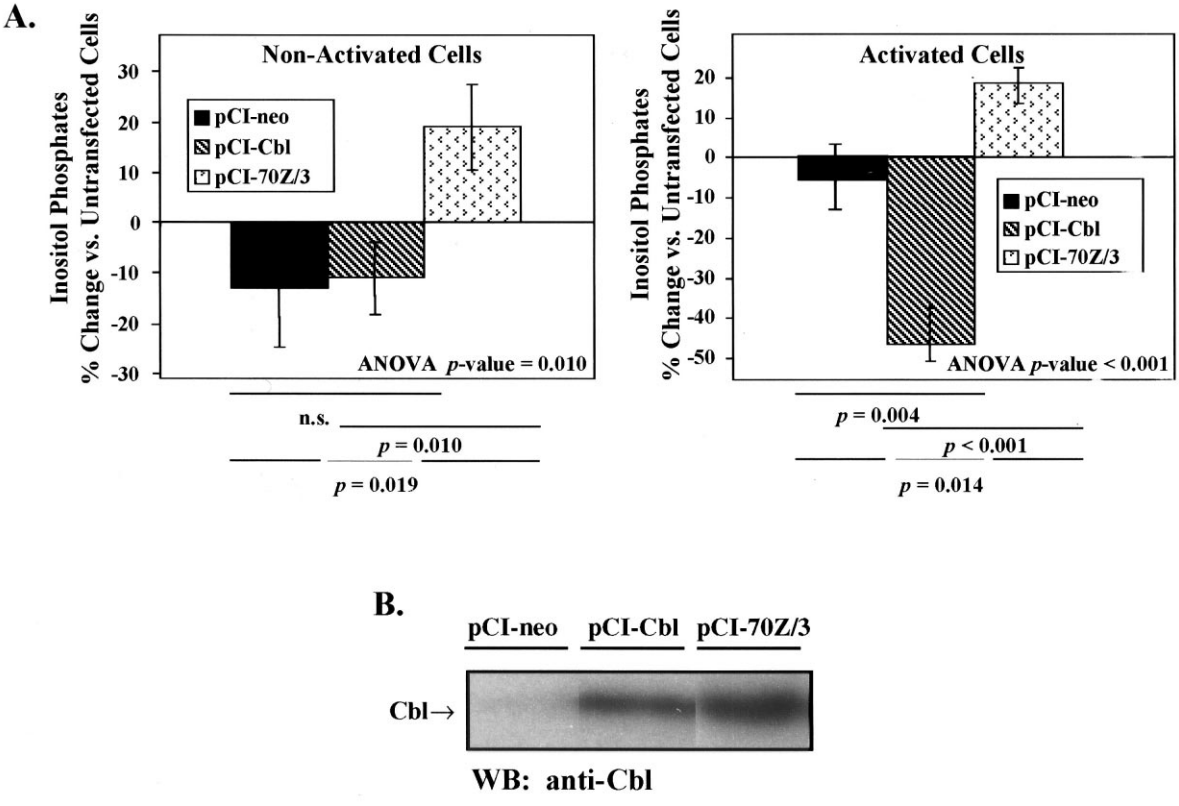

Fig. 2. Cbl over-expression inhibits TCR-induced phosphoinositide hydrolysis. A: Jurkat cells were transfected with 2 μg of the pGreenLantern™-1 expression vector along with 10 μg of pCI-neo, pCI-Cbl, or an HA-tagged pCI-70Z/3. The data were normalized as the percent change from untransfected cells. Shown is the mean ± S.D. of three experiments. B: Western blot analysis of Jurkat cells that were transfected with 20 μg pCI-neo, pCI-Cbl, or an HA-tagged pCI-70Z/3. Membranes were probed with an anti-Cbl antibody.

activator of AP-1. As shown in Fig. 1C, stimulation with CNMUT2B and PMA resulted in no inhibition of NF-AT-SEAP activity in Cbl over-expressing cells. As expected, OKT3-induced NF-AT-SEAP activity was inhibited by Cbl over-expression even in the presence of CNMUT2B because of Cbl-mediated inhibition of AP-1 activation. No PMA-inducible NF-AT activity over background was observed after transfection of a WT calcineurin construct or the vector control for CNMUT2B (data not shown). These data indicate that Cbl does not affect events regulated by calcineurin and does not directly interfere with the activity of NF-AT transcription factors.

Recently, two studies have shown that 70Z/3 Cbl augments the activity of NF-AT reporter constructs in unstimulated or ionomycin-stimulated T cells [31,32] and we have also found that 70Z/3 Cbl augments NF-AT activity in unstimulated T cells (Fig. 1D). These reports suggest that this augmentation may be due to activation of Ras and AP1 activity in these cells [31,32]. However, these reports are conflicting on the effect 70Z/3 Cbl has on OKT3-induced NF-AT activation. Although we have often seen decreases in OKT3-induced NF-AT activation upon expression of 70Z/3, our results have been variable and inconclusive (Fig. 1D and data not shown). Interestingly, an increase in PMA induced NF-AT activation can be seen in 70Z/3 expressing cells (Fig. 1D), suggesting that 70Z/3 Cbl may also be able to augment NF-AT activity by synergizing with PMA-induced Ras activation via an increase in the activity of the PLCγ1/Ca^{2+} pathway.

3.2. Cbl regulates events required for optimal PLCγ1 activity

These data demonstrate that Cbl over-expression can affect NF-AT activation by inhibiting events that lead to calcineurin activation. In addition, 70Z/3 Cbl appears to augment NF-AT activation in PMA-treated cells. We therefore questioned whether Cbl could regulate events upstream of Ca^{2+} mobilization by directly affecting PLCγ1 activation. The activation of PLCγ1 results in PI hydrolysis which generates the second messengers diacylglycerol and 1,4,5 triphosphate which control protein kinase C activity and Ca^{2+} mobilization, respectively. To directly assess PLCγ1 activation, PI hydrolysis was analyzed in the fraction of cells which were transiently transfected with Cbl. Jurkat cells were co-transfected with the pGreenLantern™-1 expression vector, which encodes a form of green fluorescent protein (GFP), and either pCI-neo or pCI-Cbl. Transfected cells were then isolated by positive sorting on a fluorescence activated cell sorter (FACS). Positive (transfected) and negative (untransfected) cells were then labeled with *myo*-[^{3}H]inositol and stimulated with OKT3 in the presence of lithium chloride to block inositol phosphate phosphatases. The results demonstrate that, while transfection with pCI-neo results in no change in PI hydrolysis, Cbl over-expression results in approximately a 40% decrease in OKT3-induced PLCγ1 activity as assessed by PI hydrolysis ($P = 0.004$) (Fig. 2A). Cbl over-expression, however, did not affect the inositol phosphate level of non-activated cells. This decrease in OKT3-induced PI hydrolysis was observed when

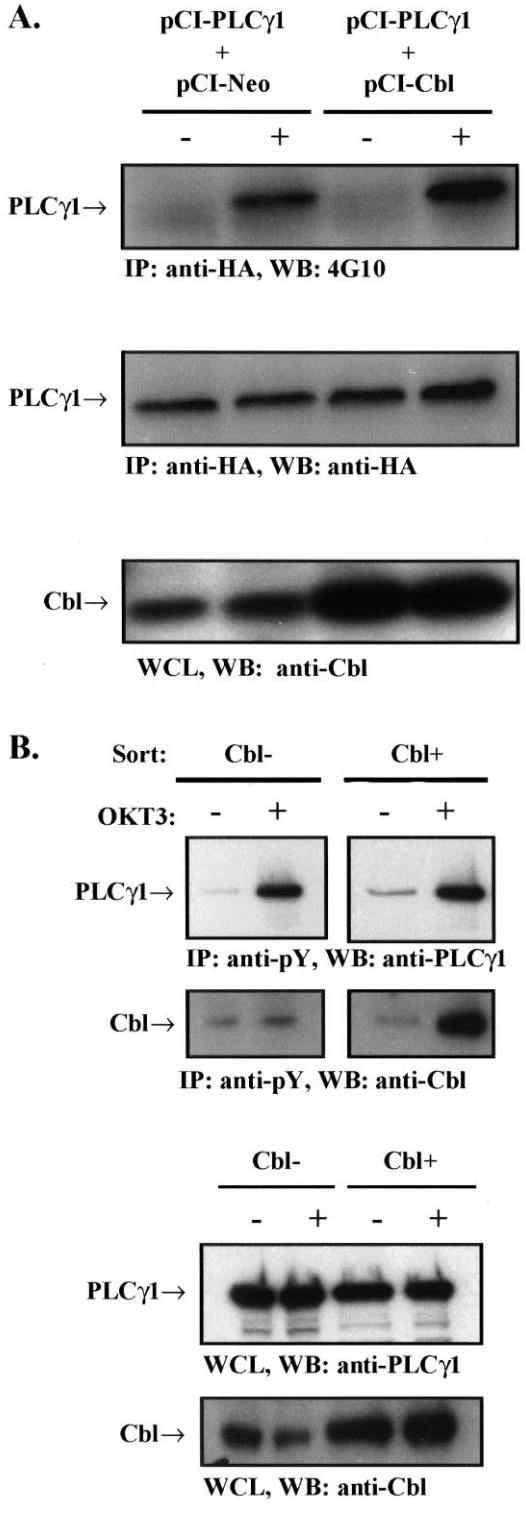

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Fig. 3. Cbl over-expression does not affect TCR-induced PLCγ1 tyrosine phosphorylation. A: Jurkat cells were transfected with 5 μg of HA-tagged pCI-PLCγ1 and 10 μg of either pCI-Cbl or pCI-neo. Cells were either left unstimulated or stimulated with OKT3 $F(ab')_2$ for 2 min. Anti-HA immunoprecipitates were resolved by SDS–PAGE and immunoblotted with an anti-phosphotyrosine antibody (4G10). The membranes were then stripped and re-probed with anti-HA. WCL (0.5×10^6/lane) were probed with anti-Cbl. B: Jurkat cells, transfected with 2 μg of pGreenLantern™-1 and 10 μg of pCI-Cbl, were sorted by FACS. Cbl-transfected (Cbl^+) and untransfected (Cbl^-) cells were left unstimulated or stimulated with OKT3. Samples were then immunoprecipitated with the anti-phosphotyrosine antibody, PY54, and immunoblotted with anti-PLCγ1 mixed monoclonal antibodies or anti-Cbl. WCL were probed with anti-PLCγ1 or anti-Cbl.

Cbl over-expressing cells were compared to either untransfected (negatively sorted) control cells or positively sorted cells transfected with the empty vector, pCI-neo. These data indicate that Cbl regulates a step that is required for optimal PLCγ1 activity.

While Cbl over-expression led to a 70% decrease in NF-AT activity, we only observed a 40% decrease in inositol phosphate generation. This discrepancy is explained by the non-linear relationship between inositol 1,4,5-trisphosphate and calcium mobilization [39]. Furthermore, there appears to be a non-linear relationship between calcium levels and calcineurin phosphatase activity as well as between calcineurin activity and IL-2 production [4]. Based on these reports, small changes in inositol phosphate generation would be predicted to lead to substantial changes in NF-AT activity.

We next investigated the effect of 70Z/3 Cbl on PLCγ1 activity. Jurkat cells were transfected with pCI-70Z/3 or pCI-neo and the pGreenLantern™-1 plasmid and sorted on the basis of GFP expression. Expression of the 70Z/3 Cbl construct resulted in a small but significant increase in PI hydrolysis in both unstimulated ($P = 0.019$) and OKT3-stimulated ($P = 0.014$) cells when compared with control cells (Fig. 2A). The expression of Cbl and 70Z/3 Cbl were comparable, ruling out that the differential effects on PLCγ1 activity were due to differences in the expression levels of each construct (Fig. 2B). These data also rule out the possibility that the inhibition of PLCγ1 activity seen with Cbl over-expression is due to a transfection artifact and suggest that 70Z/3 Cbl can augment NF-AT activation by increasing PLCγ1 activity.

3.3. Cbl over-expression has no effect on TCR-induced PLCγ1 tyrosine phosphorylation

PLCγ1 activation is a multi-step process that involves tyrosine-phosphorylation of PLCγ1 [40,41]. To determine whether Cbl over-expression was inhibiting TCR-induced tyrosine phosphorylation of PLCγ1, Jurkat cells were transiently trans-

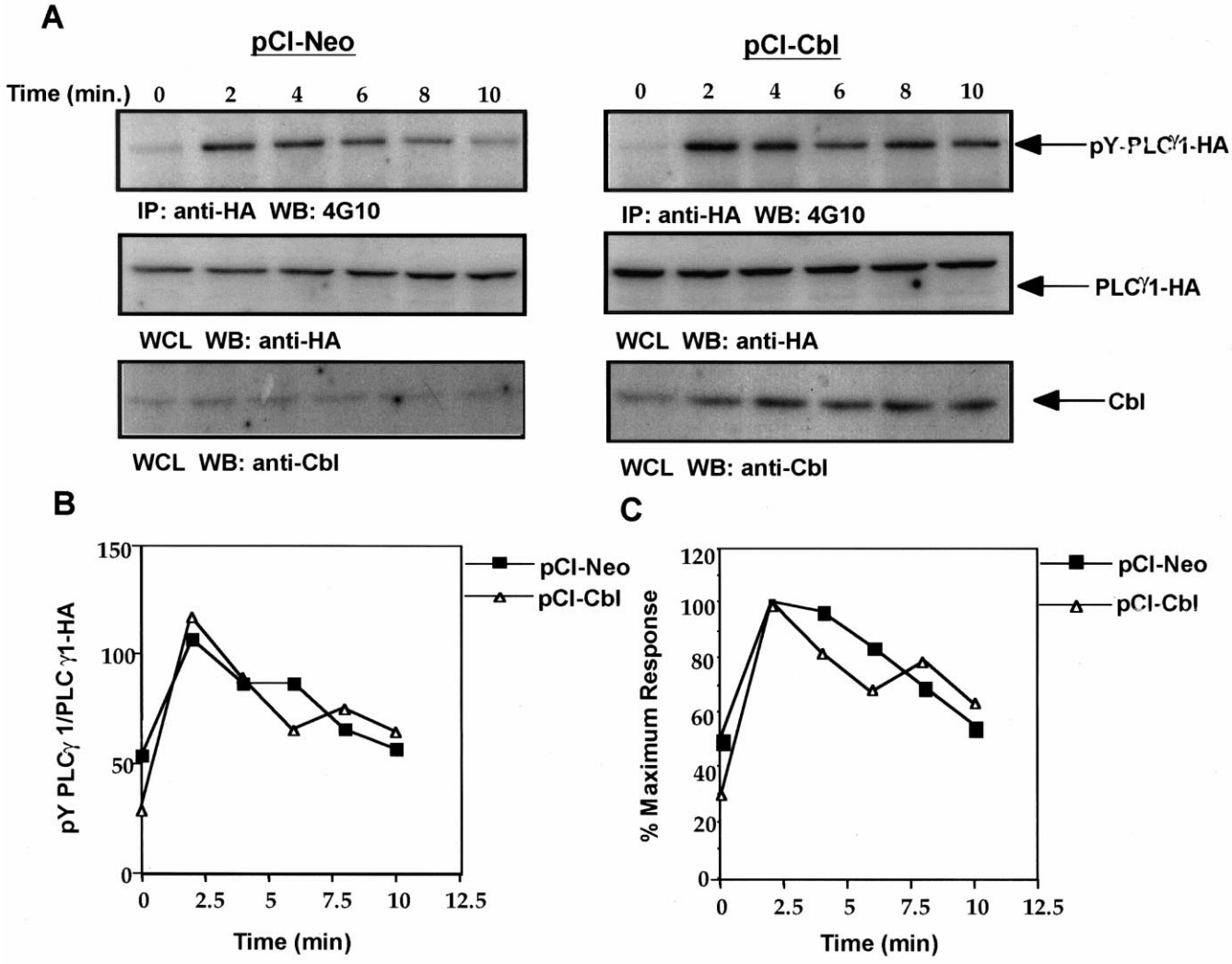

Fig. 4. Cbl over-expression does not affect the time-course of TCR-induced PLCγ1 tyrosine phosphorylation. A: Jurkat cells were transfected with 5 μg of HA-tagged pCI-PLCγ1 and 10 μg of either pCI-Cbl or pCI-neo as indicated. Cells were either left unstimulated or stimulated with OKT3 $F(ab')_2$ for the indicated amount of time. Anti-HA immunoprecipitates were resolved by SDS–PAGE and immunoblotted with an anti-phosphotyrosine antibody (4G10). WCL were first probed with anti-HA to detect transfected PLCγ1-HA. Subsequent to this they were stripped and probed for Cbl expression. The extent of PLCγ1 phosphorylation of the anti-HA immunoprecipitates was quantitated using a phosphorimage analyzer (ImageQuant Software) and normalized either to total PLCγ1-HA (B) or to peak phosphorylation seen at 2 min (C).

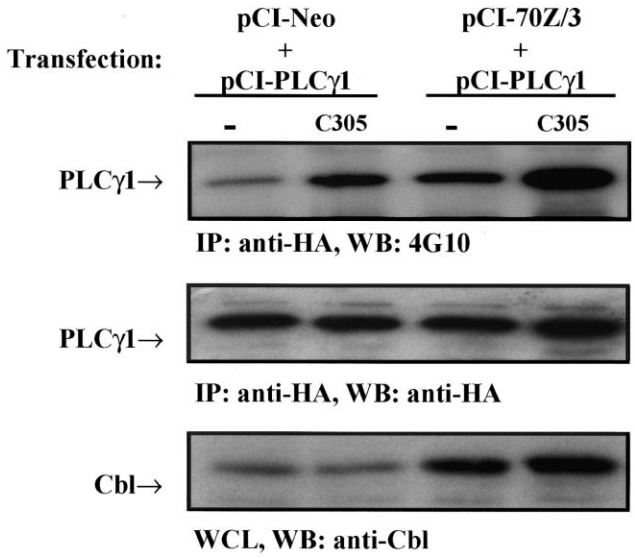

Fig. 5. 70Z/3 Cbl expression augments TCR-induced PLCγ1 tyrosine phosphorylation. Jurkat cells, transiently transfected with 10 μg of an HA-tagged PLCγ1 and 10 μg of either pCI-neo or non-HA-tagged pCI-70Z/3 Cbl, were left unstimulated or activated with the anti-TCR antibody, C305. Lysates were immunoprecipitated with anti-HA, and immunoblotted with an anti-phosphotyrosine antibody (4G10). The membrane was then stripped and re-probed with anti-HA to show the amount of immunoprecipitated PLCγ1. WCL were immunoblotted for 70Z/3 Cbl expression with an anti-Cbl antibody.

fected with a HA-tagged PLCγ1 construct (pCI-PLCγ1) and either pCI-Cbl or pCI-neo. No difference was seen in TCR-induced PLCγ1 tyrosine phosphorylation between cells over-expressing Cbl and vector control cells, as determined by anti-phosphotyrosine Western blot analysis of anti-HA immunoprecipitates from unstimulated and OKT3 stimulated cells (Fig. 3A). The over-expression of Cbl was confirmed by anti-Cbl Western blot analysis of whole cell lysates (WCL). To ensure that the over-expression of PLCγ1 in these experiments was not overcoming the ability of Cbl to regulate a step involved in PLCγ1 tyrosine phosphorylation, we also investigated the effect of Cbl on the tyrosine phosphorylation of endogenous PLCγ1. Jurkat cells were transfected with the pGreenLantern™-1 expression vector and pCI-Cbl. Transfected (GFP^+) and untransfected (GFP^-) cells were then selected by fluorescence sorting and either left unstimulated or stimulated with OKT3. The degree of PLCγ1 and Cbl tyrosine phosphorylation was determined by anti-PLCγ1 and anti-Cbl Western blot analysis of anti-phosphotyrosine immunoprecipitates (Fig. 3B). To ensure that the immunoprecipitation of PLCγ1 was not due to secondary interactions with other proteins, lysates were boiled in lysis buffer containing 1% SDS prior to dilution and immunoprecipitation. Therefore, any phosphorylated PLCγ1 immunoprecipitated under these conditions is due to a direct recognition by the immunoprecipitating antibody. As expected, Cbl over-expression resulted in a substantial increase in the amount of immunoprecipitated tyrosine phosphorylated Cbl. No difference however, was observed in the degree of PLCγ1 tyrosine phosphorylation in Cbl over-expressing cells compared to the negatively sorted, untransfected control cells (Fig. 3B). Furthermore, no difference was observed in the kinetics of PLCγ1 tyrosine phosphorylation over a 10 min time course of stimulation, at which point PLCγ1 phosphorylation has returned to near baseline levels (Fig. 4). This was true when PLCγ1 phosphorylation was calculated as a ratio with total PLCγ1-HA in WCL (Fig. 4B) or as a percent of the maximum response at 2 min (Fig. 4C). These data indicate that Cbl over-expression does not affect TCR-induced PLCγ1 tyrosine phosphorylation.

To examine the effect of expression of 70Z/3 Cbl on PLCγ1 tyrosine phosphorylation, Jurkat cells were transiently transfected with pCI-PLCγ1 and either pCI-neo or pCI-70Z/3. Anti-phosphotyrosine Western blot analysis of anti-HA immunoprecipitates was used to determine the degree of PLCγ1 tyrosine phosphorylation. The results shown in Fig. 5 demonstrate that 70Z/3 Cbl increased both resting and OKT3-stimulated PLCγ1 tyrosine phosphorylation. These results are consistent with the increase in PLCγ1 activity seen in 70Z/3 Cbl-expressing cells and indicate that 70Z/3 Cbl augments both the phosphorylation levels and activity of PLCγ1 in T cells.

4. Discussion

PLCγ1 activation requires tyrosine phosphorylation, translocation to the plasma membrane and interaction with the substrate, $PtdIns(4,5)P_2$ [40,42]. Although the events that regulate each of the steps involved in PLCγ1 activation are still unclear, activation of TCR-proximal Src kinases and ZAP-70 together with the adapters LAT and SLP-76 are required for PLCγ1 activation [36,43–45]. There are several possible mechanisms that could explain the ability of Cbl over-expression to inhibit the PLCγ/Ca^{2+} pathway. One possibility that has been previously suggested is that Cbl can negatively regulate the ZAP-70 tyrosine kinase [46]. This hypothesis is based on studies demonstrating that Cbl over-expression negatively regulates the activity of the ZAP-70 related kinase, Syk [25], that the PTB domain of Cbl binds to a negative regulatory site of ZAP-70 [47,48], that exogenous Cbl expression blocks NF-AT activation induced by ZAP-70 over-expression [47,48], and that the kinetics of phosphorylation of the ZAP-70 substrates, LAT and SLP-76 are altered in thymocytes from Cbl knockout mice [49]. However, no difference has been noted in the phosphorylation pattern or enzymatic activity of ZAP-70 after exogenous expression of Cbl or 70Z/3 Cbl [48]. In addition, our data indicate that PLCγ1 phosphorylation, which is known to be dependent on ZAP-70 activity [44], is unaffected by Cbl over-expression, arguing against an inhibition of ZAP-70 as the predominant mechanism of Cbl regulation of the PLCγ1/Ca^{2+} pathway. Furthermore, because Cbl over-expression does not alter the kinetics of PLCγ1 phosphorylation, our results also argue against the recruitment of a phosphatase as the mechanism of Cbl inhibition of the PLCγ/Ca^{2+}pathway.

Cbl appears to regulate the ligand-induced ubiquitination and degradation of the EGF receptor and the colony stimulating factor-1 (CSF-1) receptor [50,51]. This is further supported by the recent finding that the RING finger domain of Cbl can interact with UbcH7, a ubiquitin-conjugating enzyme [52]. Cbl and UbcH7 synergistically promote the ligand-induced ubiquitination of the EGF receptor, while 70Z/3 Cbl, which has a mutation in the RING finger domain and does not interact with UbcH7, reduces ligand-induced UbcH7-mediated ubiquitination of the EGF receptor [52]. Thus, Cbl may negatively regulate EGF or CSF-1 receptor signaling by promoting the ubiquitin-mediated internalization and degra-

dation of these receptors, while 70Z/3 Cbl may augment signaling by inhibition of this physiologic event [50–52].

The TCR also undergoes activation-induced ubiquitination [53]. The significance of TCR-ubiquitination has not been established and the role, if any, that Cbl plays in regulating TCR ubiquitination and degradation have yet to be examined. However, the ability of Cbl to regulate PLCγ1 activity cannot be fully explained by increased ubiquitination and degradation of signaling molecules. Although ubiquitination of TCR components can be observed within 5 min of receptor cross-linking [53], no effect of Cbl over-expression on the extent or time-course of TCR-induced PLCγ1 phosphorylation and no decrease in the total amount of PLCγ1 in WCL was observed. Although these data do not rule out a role for Cbl in the ubiquitination of TCR components, they indicate that the decrease in PLCγ1 activity observed in Cbl over-expressing cells is not due to increased degradation of PLCγ1 or other proteins required for its phosphorylation.

We have previously suggested that Cbl may participate in or regulate the formation of a multi-protein activation complex and that the over-expression of Cbl in T cells disrupts the physiologic stoichiometry of this complex leading to the formation of incomplete complexes that are incapable of activating downstream signaling pathways [28]. If Cbl regulates the formation of an activation complex, it is doing so at a point that does not affect the ability of PLCγ1 to associate with the specific kinase(s) that phosphorylates it. We have recently shown that the amino-terminal Src-homology (SH) 2 domain (SH2 (N) domain) of PLCγ1 is required for both immunoreceptor-induced phosphorylation and activation of PLCγ1, while its carboxy-terminal SH2 (SH2 (C)) and SH3 domains are required for full activation, but are dispensable for TCR-induced PLCγ1 phosphorylation [36,54]. Interestingly, Cbl constitutively interacts with GST-fusion proteins encompassing the SH3 domain of PLCγ1 [24], and in a phosphorylation-dependent manner with GST-fusion proteins encompassing the PLCγ1 SH2 domains [9]. Cbl-mediated blocking of the function of either the PLCγ1 SH3 or SH2(C) domain could result in a decrease in the activity of PLCγ1, without altering its phosphorylation pattern. We are currently investigating whether the ability of Cbl to interact with any of the SH-domains of PLCγ1 is required for its ability to regulate PLCγ1activation.

Interestingly, recent studies on another cbl family member, cbl-b, indicate that cbl-b does not regulate either the PLCγ1 or the Ras pathway. Instead it appears to be a negative regulator of Vav1 phosphorylation [55,56]. These data, taken together with those from our laboratory, suggest that different Cbl family members may regulate unique signaling pathways. Future studies on the differential ability of Cbl proteins to regulate Vav1 activation will be needed to confirm this hypothesis.

In addition to 70Z/3 Cbl, v-Cbl has been shown to be oncogenic [7,57] and a third Cbl variant was recently described as being involved in gastrointestinal tumorigenesis [58]. The oncogenic potential of these Cbl variants may be due to their ability to compete with the physiologic inhibitory function of endogenous Cbl [48]. Alternately, the transforming forms of Cbl may induce a positive signal in a non-competitive manner [31], possibly by inducing the activation of signaling steps unique from those regulated by WT Cbl. This latter mechanism is suggested by the unique effect of 70Z/3 Cbl in increasing PLCγ1 phosphorylation in both unstimulated and OKT3-stimulated T cells, compared to the lack of effect that over-expression of WT Cbl had on PLCγ1 phosphorylation. Further studies will elucidate the signaling events associated with 70Z/3 Cbl as they pertain to the mechanism of transformation by this and other Cbl variants.

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