The Cbl proto-oncogene product is a complex adapter protein that functions as a negative regulator of protein tyrosine kinases. It is rapidly tyrosine-phosphorylated and associates with Crk(L) and p85 phosphatidylinositol 3-kinase (PI3K) upon engagement of numerous receptors linked to tyrosine kinases. Elucidation of the mechanism(s) underlying Cbl deregulation is therefore of considerable interest. The 70Z Cbl oncoprotein shows increased baseline tyrosine phosphorylation in fibroblasts and enhances nuclear factor of activated T cells (NFAT) activity in Jurkat T cells. Its transforming ability has been proposed to relate to its increased phosphotyrosine content. We demonstrate that 70Z Cbl shows increased basal and activation-induced tyrosine phosphorylation and association with Crk(L) and p85 PI3K in Jurkat T cells. 70Z Cbl, however, retains the ability to enhance NFAT and activating protein 1 (AP1) activity in the absence of Crk(L)/p85 PI3K association. In contrast, the G306E mutation, which inactivates the phosphotyrosine binding domain of Cbl, blocks NFAT/AP1 activation by 70Z Cbl. We conclude that 70Z Cbl-induced NFAT/AP1 activation requires the phosphotyrosine binding domain but not Crk(L)/p85 PI3K association. We hypothesize that 70Z Cbl acts as a dominant negative by blocking the negative regulatory function of the Cbl phosphotyrosine binding domain on protein-tyrosine kinases.

The proto-oncogene product c-Cbl is a ubiquitously expressed complex adapter protein that associates with numerous signaling molecules in a variety of cell types (reviewed in Ref. 1). It was originally identified as a viral oncogene product (v-Cbl) that causes B-lymphomas and myeloid leukemias in mice (2). Cloning of its cellular homolog revealed that c-Cbl is a 906-amino acid protein that lacks any obvious catalytic domain (3). Cloning of its cellular homolog revealed that c-Cbl is a 906-amino acid protein that lacks any obvious catalytic domain (3). It contains a N-terminal phosphotyrosine binding (PTB)1 domain (4, 5), a C3HC4 ring finger motif (6), a proline-rich region that includes a binding site for the Grb2 SH3 domain (7), and a C-terminal region that includes several tyrosine residues located within consensus binding sequences for the SH2 domain-containing Crk and p85 PI3K adapter proteins (8). A Cbl-related molecule called Cbl-b has been cloned in humans (9), and Cbl homologs have been identified in Drosophila melanogaster (D-Cbl) (10, 11) and Caenorhabditis elegans (Sli-1) (12).

Cbl is rapidly tyrosine-phosphorylated in response to engagement of numerous receptors that activate protein-tyrosine kinases, including immunoreceptors, receptor protein-tyrosine kinases, hematopoietic growth factor receptors, and integrins, as well as oncogenic tyrosine kinases. Tyrosine-phosphorylated Cbl associates with the SH2 domains of the adapter proteins Crk(L) and/or p85 PI3K upon engagement of many of these receptors (reviewed in Ref. 1). Similar to Grb2, Crk family adapter proteins are composed almost exclusively of SH2 and SH3 domains. CrkI and CrkII are alternatively spliced products of a single gene that contain a N-terminal SH2 domain followed by one or two SH3 domains, respectively (13). CrkL is the product of a different gene; it has a structural organization similar to CrkII (14). Several studies have indicated that Crk(L) proteins may promote transformation, either when expressed as truncated isoforms or when overexpressed (13, 15, 16). Moreover, a multimolecular complex of Abl, CrkL, c-Cbl, and, possibly, p85 PI3K has been postulated to mediate, at least in part, the transforming ability of oncogenic Abl (16–19). Interestingly, Crk(L) proteins associate through their N-terminal SH3 domain with C3G (20), a guanine nucleotide exchange factor for the small GTPase Rap1 (21, 22). In various model systems, overexpression of Rap1 can antagonize Ras signaling, presumably through competitive inhibition of Ras-GTP binding to downstream effector molecules (Ref. 23 and references therein). Thus, it is possible that the interaction of Cbl with Crk(L) regulates Ras signaling through C3G and Rap1 and that Cbl serves as a docking protein to recruit the Crk(L)-C3G complex (24). This is analogous to the well described role of activated receptor tyrosine kinases, which recruit the Grb2-Sos complex to the plasma membrane to activate membrane-bound Ras (25). Elucidation of the functional significance of the Cbl-Crk(L)/p85 PI3K interaction for normal as well as oncogenic protein-tyrosine kinase signaling pathways is therefore of considerable interest.

Several lines of evidence indicate that Cbl functions as a negative regulator of protein-tyrosine kinase signaling pathways. First, in the flatworm C. elegans, the G315E loss-of-function allele of Sli-1 rescues vulval development induced by a reduction-of-function allele of the Let23 epidermal growth factor receptor homolog (12). Second, in D. melanogaster, overexpression of D-Cbl in transgenic flies inhibits the sevenless...
Expression of Recombinant Vaccinia Virus—Recombinant vaccinia virus was made by standard procedures. Briefly, near confluent CV-1 cells were infected in 25-cm² flasks for 2 h with wild-type WR strain TK vaccinia virus at a multiplicity of infection of 0.25 and transfected overnight with 26 µg of the appropriate constructs using LipofectAMINE Opti-MEM medium (Life Technologies, Inc.) followed by an additional 24 h of culture in Dulbecco’s modified Eagle’s medium/10% FBS. Infected/transfected cells were harvested by centrifugation and lysed by repeated cycles of freeze-thawing and sonication. Black recombinant TK plaques were purified by three rounds of plaque purification on confluent HuTK cells in 1% low melting agarose/1% basal medium Eagle (Life Technologies, Inc.)/5% FBS and three rounds of amplification in Dulbecco’s modified Eagle’s medium/10% FBS in the continuous presence of 25 µg/ml BrdUrd (Sigma). Crude viral stocks were titered on HuTK cells and used to infect Jurkat T cells at a multiplicity of infection of 5. After 18 h, infected Jurkat T cells were harvested. Cell viability was routinely determined by trypan blue exclusion and always exceeded 95%. OET3 Stimulation, Immunoprecipitation, SDS-PAGE, and Immunoblotting—Jurkat T cells were washed once in ice-cold RPMI medium without FBS and resuspended at 1 × 10⁶ cells/ml. Generally, 1 – 10⁷ cells were preincubated at 37 °C for 5 min, before cross-linking CD3 by addition of OET3 ascites (1:100). Cells were incubated at 37 °C for the indicated time periods and solubilized for 30 min on ice in lysis buffer containing 150 mM NaCl, 25 mM Tris, pH 7.5, 1 mM NaVO₄, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml apotinin and leupeptin, and 1% Brij97 or another detergent as indicated. Immunoprecipitation of postnuclear lysates, denaturing SDS-PAGE, and immunoblotting were performed according to standard procedures.

Transient Transfection and SEAP Reporter Gene Assays—Secreted alkaline phosphatase (SEAP) reporter gene constructs (5) were a kind gift of multimers of the NFA or AP1 axis (NFAT-SEAP and AP1-SEAP) were kindly provided by G. Crabtree (32). In general, 1 × 10⁵ Jurkat-TAg cells were transfected with 5 µg of reporter construct and 10 µg of test construct by electroporation using the Bio-Rad gel pulser (310 kV, 200 ohms, 960 microfarad). Transfected Jurkat-TAg cells were cultured in bulk for 24 h and subsequently stimulated in duplicate with immobilized OKT3 (1 µg of ascites/well), PMA (Sigma) at 10 ng/ml, ionomycin (Calbiochem) at 1 µg/ml or PMA plus ionomycin in 1 ml of phenol red-deficient RPMI/10% FBS at a density of 3 × 10⁶ transfected cells/ml. After stimulation for 15 h, cell cultures were incubated at 1 h at 65 °C to inactivate endogenous phosphatases, and supernatants were assayed in duplicate at 37 °C for SEAP activity using p-nitrophenyl phosphate (Sigma) at 1.8 mg/ml in diethanolamine bicarbonate, pH 10.0, as a substrate. Absorbance at 405 nm was determined using a MR 5000 microtiter plate reader (Dynatech), usually between 6 and 12 h of incubation. Presented data are representative of at least three independent experiments.

RESULTS

70Z Cbl Shows Increased Basal and Activation-induced Tyrosine Phosphorylation and Association with Crk(L) and p85 PI3K in Jurkat T Cells—The oncogenic 70Z/B mutant form of Cbl undergoes increased baseline tyrosine phosphorylation in fibroblasts (28–30). To determine whether increased tyrosine phosphorylation of 70Z Cbl could also be observed in T cells, Jurkat T cells were infected with recombinant vaccinia virus
Jurkat T cells. Taken together, these findings demonstrate that increased basal and activation-induced tyrosine phosphorylation of 70Z relative to wt Cbl leads to increased association with Crk(L) and p85 PI3K adapter proteins.

**Fig. 1.** 70Z Cbl shows increased basal and CD3-induced tyrosine phosphorylation in Jurkat T cells. A, Jurkat T cells were infected for 15 h with recombinant vaccinia virus generated with vector alone (pSC65) or vector encoding HA-tagged wt Cbl (HA Cbl) or 70Z Cbl (HA 70Z). Cells were stimulated for 2 min in the presence or absence of OKT3 mAb and solubilized in 1% Brij97 lysis buffer, and postnuclear lysates were immunoprecipitated (IP) with anti-HA mAb, followed by SDS-PAGE and sequential immunoblotting (IB) with anti-phosphotyrosine (4G10) and anti-HA mAbs as indicated. The band detected in the anti-HA immunoblot (lower panel) of pSC65 infected cells that migrates just above Cbl is a background band caused by HA blotting and does not represent Cbl, as it is not detected by anti-Cbl blotting (see also Fig. 3). B, after infection with either HA Cbl or HA 70Z, Jurkat T cells were stimulated with OKT3 mAb for the indicated time periods and lysed in 1% Brij97 lysis buffer, and postnuclear lysates were immunoprecipitated (IP) with anti-HA mAb followed by SDS-PAGE and sequential immunoblotting (IB) with anti-phosphotyrosine (4G10) and anti-Cbl (C15) Abs as indicated.

**Fig. 2.** 70Z Cbl proteins show increased basal and CD3-induced association with Crk(L) adapter proteins. Jurkat T cells were infected, stimulated with OKT3, and solubilized as in Fig. 1A. Postnuclear lysates were immunoprecipitated (IP) with anti-CrkII, anti-CrkL, or anti-HA Abs followed by SDS-PAGE and (sequential) immunoblotting (IB) with anti-HA and anti-Crk, anti-HA and anti-CrkL, or anti-HA, respectively.

| Vaccinia: | OKT3: | pSC65 | HA Cbl | HA 70Z |
|----------|-------|-------|--------|--------|
| Cbl      | -     | +     | -      | +      |
| CrkII    | -     | -     | +      | -      |
| CrkL     | -     | -     | -      | +      |

**Table 1.** Kinetics of Tyrosine Phosphorylation of 70Z Cbl Proteins in Jurkat T Cells.

| Vaccinia: | OKT3 (min): | HA Cbl | HA 70Z |
|----------|-------------|--------|--------|
| 0        | 2           | 5      | 10     | 20     | 40     |
| IP: HA   | IB: 4G10    |        |        |        |        |
| IP: HA   | IB: HA      |        |        |        |        |
| IP: HA   | IB: HA      |        |        |        |        |

**Table 2.** Tyrosine Phosphorylation of 70Z Cbl Proteins in Response to OKT3 Stimulation.

| Vaccinia: | OKT3: | pSC65 | HA Cbl | HA 70Z |
|----------|-------|-------|--------|--------|
| Cbl      | -     | +     | -      | +      |
| CrkII    | -     | -     | +      | -      |
| CrkL     | -     | -     | -      | +      |

**Table 3.** Association of 70Z Cbl Proteins with Crk(L) and p85 PI3K Adapter Proteins.

| Vaccinia: | OKT3: | pSC65 | HA Cbl | HA 70Z |
|----------|-------|-------|--------|--------|
| Cbl      | -     | +     | -      | +      |
| CrkII    | -     | -     | +      | -      |
| CrkL     | -     | -     | -      | +      |
| IP: HA   | IB: HA |        |        |        |
| IP: HA   | IB: HA |        |        |        |
| IP: HA   | IB: HA |        |        |        |

**Table 4.** Summary of Tyrosine Phosphorylation in Response to OKT3 Stimulation.

| Vaccinia: | OKT3: | pSC65 | HA Cbl | HA 70Z |
|----------|-------|-------|--------|--------|
| Cbl      | -     | +     | -      | +      |
| CrkII    | -     | -     | +      | -      |
| CrkL     | -     | -     | -      | +      |
| IP: HA   | IB: HA |        |        |        |
| IP: HA   | IB: HA |        |        |        |
| IP: HA   | IB: HA |        |        |        |

**Table 5.** Association of 70Z Cbl Proteins with Crk(L) and p85 PI3K Adapter Proteins.

| Vaccinia: | OKT3: | pSC65 | HA Cbl | HA 70Z |
|----------|-------|-------|--------|--------|
| Cbl      | -     | +     | -      | +      |
| CrkII    | -     | -     | +      | -      |
| CrkL     | -     | -     | -      | +      |
| IP: HA   | IB: HA |        |        |        |
| IP: HA   | IB: HA |        |        |        |
| IP: HA   | IB: HA |        |        |        |

**Table 6.** Summary of Tyrosine Phosphorylation in Response to OKT3 Stimulation.

| Vaccinia: | OKT3: | pSC65 | HA Cbl | HA 70Z |
|----------|-------|-------|--------|--------|
| Cbl      | -     | +     | -      | +      |
| CrkII    | -     | -     | +      | -      |
| CrkL     | -     | -     | -      | +      |
| IP: HA   | IB: HA |        |        |        |
| IP: HA   | IB: HA |        |        |        |
| IP: HA   | IB: HA |        |        |        |

**Table 7.** Association of 70Z Cbl Proteins with Crk(L) and p85 PI3K Adapter Proteins.

| Vaccinia: | OKT3: | pSC65 | HA Cbl | HA 70Z |
|----------|-------|-------|--------|--------|
| Cbl      | -     | +     | -      | +      |
| CrkII    | -     | -     | +      | -      |
| CrkL     | -     | -     | -      | +      |
| IP: HA   | IB: HA |        |        |        |
| IP: HA   | IB: HA |        |        |        |
| IP: HA   | IB: HA |        |        |        |
and data not shown), it seems that these additional tyrosine residues are located in the C-terminal region (amino acids 655–906) of Cbl. Consistent with this idea, Cbl contains four additional tyrosine residues in that region, i.e. Tyr-674, Tyr-735, Tyr-869, and Tyr-871 (3).

**Disruption of Crk(L) and p85 PI3K Association with wt and 70Z Cbl Carrying the Y700F/Y731F/Y774F Triple Mutation**—To further characterize the molecular basis for the interaction between Cbl and Crk(L)/p85 PI3K proteins in *vivo*, we analyzed the interaction of Myc epitope-tagged CrkI, as well as endogenous CrkII and CrkL with wt or 70Z Cbl carrying Y700F, Y731F, and/or Y774F mutations. Recombinant vaccinia virus carrying Myc epitope-tagged CrkI was generated for this purpose. As previously mentioned, Myc CrkI displayed increased association with 70Z relative to wt Cbl in both unstimulated and anti-CD3-stimulated Jurkat T cells (Fig. 4A, compare lanes 3 and 4 with lanes 9 and 10). Furthermore, both Y700F and Y774F single point mutations reduced, but did not eliminate, binding of CrkI to wt and 70Z Cbl (Fig. 4A, compare lanes 5–8 with lanes 3 and 4 and lanes 11–14 with lanes 9 and 10). Identical results were obtained also for coprecipitation of wt and 70Z Cbl with endogenous CrkII and CrkL (data not shown). In contrast, wt and 70Z Cbl carrying the double (Y700F/Y774F) or triple (Y700F/Y731F/ Y774F) mutation failed to show basal and activation-induced association with CrkI (Fig. 4B, compare lanes 5–8 with lanes 3 and 4 and lanes 11–14 with lanes 9 and 10), as well as with endogenous CrkII and CrkL (data not shown). Additional experiments using Myc epitope-tagged Crk proteins that contain a mutation that inactivates its SH2 (R38V) or SH3 (W169L) domain confirmed that the interaction between Cbl and Crk is mediated through the SH2 but not the SH3 domain of Crk in *vivo* (data not shown).

We also analyzed the interaction of p85 PI3K with various wt and 70Z Cbl constructs. Initial experiments revealed that following solubilization in Brij97, p85 coprecipitated via the OKT3 mAb with the activated T cell receptor/CD3 complex (data not shown). To exclude the possibility that p85 PI3K could be detected in anti-HA immunoprecipitates as a consequence of its association with the activated TCR/CD3 complex rather than Cbl proteins, we instead used Triton X-100 to solubilize the cells. As shown in Fig. 5A, we could not detect activation-induced coprecipitation of p85 PI3K in anti-HA immunoprecipitates of vector infected Jurkat T cells under these conditions (Fig. 5A, lanes 1 and 2). We did detect a faint background band in both unstimulated and anti-CD3-stimulated vector-infected Jurkat T cells that comigrated with p85 PI3K. Nevertheless, we clearly observed increased p85 PI3K binding to wt Cbl in anti-CD3-stimulated relative to unstimulated HA Cbl infected Jurkat T cells (Fig. 5A, compare lanes 3 and 4 with lanes 1 and 2), as well as increased basal and activation-induced p85 PI3K association with 70Z relative to wt Cbl (Fig. 5A, compare lanes 7 and 8 with lanes 3 and 4). Significantly, anti-HA immunoblotting of anti-p85 PI3K immunoprecipitates more clearly revealed increased association of p85 PI3K with 70Z relative to wt Cbl when compared with anti-p85 PI3K immunoblotting of anti-HA immunoprecipitates (compare Fig. 5B and Fig. 5A). Even though p85 PI3K was readily detected in anti-HA immunoprecipitates of HA Cbl expressing cells (Fig. 5A, lanes 3 and 4), activation-induced coprecipitation of HA-tagged Cbl with p85 PI3K could barely be detected (Fig. 5B, lanes 3 and 4). These findings are consistent with the possibility that overexpressed HA Cbl associates with a small fraction of total p85 PI3K, whereas the overexpressed and heavily tyrosine-phosphorylated 70Z Cbl protein associates with a large fraction of total p85 PI3K. Importantly, and consistent with published data (24, 31, 37), p85 PI3K did not associate with wt or 70Z Cbl carrying the Y700F/Y731F/Y774F triple mutation (Figs. 5, A and B, compare lanes 5 and 6 with lanes 3 and 4, and lanes 9 and 10 with lanes 7 and 8). Taken together, our findings demonstrate that Crk(L) proteins interact through their SH2 domains with both phosphoryrosine residues 700 and 774 in wt and 70Z Cbl proteins *in vivo*, and that simultaneous mutation of Tyr-700, Tyr-731, and Tyr-774 disrupts interaction of Crk(L) and p85 PI3K with wt and 70Z Cbl.

**Disruption of Crk(L)/p85 PI3K Interaction with wt and 70Z Cbl Does Not Affect Erk MAPK Activation**—Crk(L) proteins associate through their N-terminal SH3 domain with C3G (20), a guanine nucleotide exchange factor for the small G protein Rap1 (21). Overexpression of Crk(L) and C3G has been reported to activate Rap1 (38). In various model systems, overexpression of Rap1 can antagonize Ras signaling, presumably through competitive inhibition of Ras-GTP binding to downstream effector molecules (Ref. 23 and references therein). In Jurkat T cells, activation of the Ras effector protein Raf results in activation of the dual specificity MAP kinase kinases MEK1/2 that, in turn, activate the MAP kinases Erk1 and Erk2. Thus, Jurkat T cells were infected with recombinant vaccinia virus expressing the various wt and 70Z Cbl proteins, and activation of Erk1/2 was evaluated by immunoblotting whole cell lysates with anti-active MAPK Ab. Neither overexpression of 70Z Cbl nor overexpression of the Y700F/Y774F or Y700F/Y731F/Y774F mutant derivatives of wt and 70Z Cbl showed any significant and reproducible effect on basal or anti-CD3-induced activation of Erk1/2 relative to wt Cbl or the vector control (Fig. 6). Moreover, none

![Figure 3](https://via.placeholder.com/150)
of the wt or 70Z Cbl proteins had any effect on basal or anti-CD3-induced Erk activation upon prolonged stimulation (data not shown), excluding the possibility that 70Z Cbl induces prolonged Erk activation relative to wt Cbl or the vector control. These results demonstrate that increased basal and activation-induced association of 70Z Cbl with Crk(L) and p85 PI3K does not detectably affect Erk1/2 activation, suggesting that the Cbl-Crk(L) and Cbl-p85 PI3K interactions do not regulate Ras signaling in Jurkat T cells.

70Z Cbl Retains Its Ability to Induce NFAT and AP1 in the Absence of Crk(L)/p85 PI3K Association—As 70Z Cbl is known to activate the NFAT transcription factor in Jurkat T cells (31), we analyzed the effect of the Cbl-Crk(L) and Cbl-p85 PI3K interactions on transcriptional activation of NFAT and AP1 transcription factors using SEAP reporter gene assays (32). Jurkat-TAg cells were transiently transfected with HA-tagged wt or 70Z Cbl expression constructs together with the appropriate reporter gene construct. Transfected cells were left unstimulated or stimulated with immobilized anti-CD3 mAb, PMA, ionomycin or PMA plus ionomycin, and supernatants were assayed for SEAP reporter gene activity. Results were expressed relative to the response obtained after stimulation with PMA plus ionomycin, which served as an internal control for the SEAP responsiveness between different groups of transfected cells. It should be noted that we did not observe any reproducible effect of overexpressing various Cbl proteins on the absolute response induced by PMA plus ionomycin (data not shown). Overexpression of wt Cbl did not reproducibly and significantly affect NFAT activity under any conditions relative to the vector control (Fig. 7A), nor did overexpression of wt Cbl affect AP1 driven reporter gene activity (Fig. 7B). In contrast, overexpression of 70Z Cbl led to a significant and reproducible increase in NFAT reporter activity in unstimulated cells relative to wt Cbl or the vector control but caused no significant and reproducible changes in response to anti-CD3 mAb (Fig. 7A). Indeed, titration of the anti-CD3 mAb over a 100-fold range did not reveal any significant effect of 70Z relative to wt Cbl on anti-CD3-induced NFAT activation (data not shown). 70Z Cbl also up-regulated AP1 activity in unstimulated cells, although this increase was less pronounced (2–3-fold induction over the vector control) compared with the increase in NFAT activity (5–10-fold induction over the vector control) (Fig. 7B). Overexpression of wt or 70Z Cbl Y700F/Y774F double or Y700F/Y731F/Y774F triple mutants did not lead to significant changes in NFAT or AP1 activity relative to their unmutated counterparts (Fig. 7, A and B). It should be noted that the relatively small increase in NFAT activation induced by 70Z Y700F/Y774F and 70Z Y700F/Y731F/Y774F triple mutants did not lead to significant changes in NFAT or AP1 activity relative to their unmutated counterparts (Fig. 7, A and B). It should be noted that the relatively small increase in NFAT activation induced by 70Z Y700F/Y774F and 70Z Y700F/Y731F/Y774F relative to 70Z Cbl observed in this experiment was not consistently observed. In contrast to the reported cooperation of 70Z Cbl with ionomycin to induce NFAT (31), our results demonstrated that SEAP reporter gene activity in cells stimulated with ionomycin (Fig. 7, A and B) or PMA alone (data not shown) was similar to that observed in unstimulated cells. It should be noted that the observed effects of oncogenic 70Z Cbl proteins on NFAT and AP1 activation were not due to differences in expression levels of Cbl proteins, as evidenced by anti-Cbl immunoblotting of whole cell lysates (Fig. 7C). Taken together, our results demonstrate that i) 70Z Cbl up-regulates NFAT and AP1 activity in unstimulated Jurkat T cells, ii) 70Z Cbl does not cooperate with ionomycin to induce NFAT or AP1 activity, and iii) the 70Z Cbl-induced activation of NFAT and AP1 is not mediated through its interaction with Crk(L) and p85 PI3K adapter proteins.
Disruption of the PTB Domain Blocks 70Z Cbl-induced NFAT/AP1 Activation—Thien and Langdon (27, 30) have hypothesized that the mechanisms underlying 70Z Cbl- and v-Cbl-induced transformation are distinct. Specifically, they hypothesized that 70Z Cbl induced transformation results from a positive signal, perhaps related to its increased tyrosine phosphorylation (29, 30), whereas v-Cbl appears to act as a dominant negative by competing with endogenous Cbl for phosphorytrosine residues on activated tyrosine kinases, thereby blocking the putative negative regulatory role of Cbl (27). Such a model appears to be supported by the observation that 70Z Cbl, but not v-Cbl, enhances NFAT activation in Jurkat T cells (Ref. 31 and this study), but the molecular mechanisms underlying 70Z Cbl- and v-Cbl-induced transformation are different from each other and presently not available. As v-Cbl-induced transformation is blocked by the G306E mutation (27), we analyzed the effect of the G306E mutation on NFAT and AP1 activation induced by 70Z Cbl oncoproteins. Jurkat TAG cells were transiently transfected with HA-tagged 70Z Cbl in either the absence or the presence of the G306E mutation. Activation of NFAT and AP1 transcription factors was assessed in unstimulated Jurkat T cells. Most importantly, the 70Z Cbl oncoprotein but not its G306E mutant derivative up-regulated NFAT and AP1 activity (Fig. 8, A and B), even though 70Z Cbl and its G306E mutant derivative were expressed at similar levels (Fig. 8C). As the PTB domain of Cbl is known to bind to the ZAP70 phosphotyrosine residue 292 in vitro and upon coexpression in Cos cells (4, 5), we next evaluated whether the G306E mutation might also affect tyrosine phosphorylation of the 70Z Cbl oncoprotein. As illustrated in Fig. 8D, the increased tyrosine phosphorylation of 70Z Cbl that is observed in unstimulated and OKT3-stimulated Jurkat T cells is blocked by the G306E mutation and is comparable to that observed in wt Cbl. Taken together, our findings confirm earlier reports that c/v-Cbl does not up-regulate NFAT and AP1 activity (31) and demonstrate that increased tyrosine phosphorylation of 70Z Cbl-induced as well as 70Z Cbl-induced NFAT and AP1 activation requires the presence of an intact PTB domain.

DISCUSSION

The Cbl proto-oncogene product is a ubiquitously expressed complex adapter protein that functions as a negative regulator of protein-tyrosine kinases (1, 11, 12, 26). Cbl is rapidly tyrosine-phosphorylated and associates with Crk(L) and p85 PI3K adapter proteins upon engagement of numerous protein-tyrosine kinase-linked receptors in a variety of cell types (reviewed in Ref. 1). Interestingly, the 70Z Cbl oncoprotein shows increased tyrosine phosphorylation in fibroblasts (28–30) and activates NFAT-mediated transcription in Jurkat T cells (Ref. 31 and this study), but the molecular mechanisms underlying...
70Z Cbl-induced NFAT activation and transformation have not been previously identified. Here we demonstrate that 70Z Cbl shows increased basal and CD3-induced tyrosine phosphorylation, leading to increased association with Crk(L) and p85 PI3K adapter proteins in Jurkat T cells. However, disruption of Crk(L) and p85 PI3K association with oncogenic 70Z Cbl did not block NFAT and AP1 activation. In contrast, 70Z Cbl-induced NFAT/AP1 activation was completely blocked by the G306E mutation, indicating that 70Z Cbl requires an intact PTB domain for NFAT/AP1 activation in Jurkat T cells.

Our studies confirm the previous finding (31) that oncogenic 70Z Cbl but not wt or c/v-Cbl activates NFAT and further extends these findings to show that 70Z Cbl also enhances AP1 activity. Our study differs from that of Liu et al. (31) in that we did not detect cooperation of 70Z Cbl with ionomycin to induce NFAT activation, even when Jurkat T cells were stimulated after serum starvation or when using lower concentrations of ionomycin (data not shown). We also did not confirm the data from Rellahan et al. (40), who reported a 3-fold reduction in AP1-mediated reporter activity in cells overexpressing Cbl rel-

**Fig. 7. Activation of NFAT and AP1 by oncogenic 70Z Cbl in unstimulated Jurkat T cells does not require interaction with Crk(L) and p85 PI3K.** Jurkat-TAg cells were transiently transfected with vector (pSX SRe) or the indicated Cbl expression constructs together with the NFAT (A) or AP1 (B) reporter gene construct and either left unstimulated or stimulated for 15 h with immobilized OKT3, ionomycin (1 μg/ml), or PMA (10 ng/ml) plus ionomycin. SEAP reporter activity was measured and plotted relative to the response induced by PMA plus ionomycin. C, whole cell lysates (WCL) of the experiments shown in A and B were immunoblotted (IB) for Cbl expression using anti-Cbl (C15) Abs.
ative to the vector control. The cause of the discrepancies between our study and these other studies is not clear at present, but we note that we used PMA plus ionomycin stimulation as an internal control to normalize for reporter gene activity between different groups of transfected cells.

The 70Z Cbl oncoprotein shows increased baseline tyrosine phosphorylation in fibroblasts (28–30) and, as demonstrated in this study, in Jurkat T cells. However, the tyrosine residues in 70Z Cbl that undergo increased phosphorylation relative to wt Cbl have not been identified thus far. In vitro studies have previously identified Tyr(P)-774 and Tyr(P)-731 in wt Cbl as binding sites for the SH2 domains of Crk and p85 PI3K, respectively (24, 36). In vivo studies have demonstrated a role for both Tyr(P)-700 and Tyr(P)-774 in CrkL binding in Abl transformed cells (33) and for Tyr(P)-731 in p85 PI3K binding (31, 37). Our studies have confirmed and further extended these findings in determining that CrkI, CrkII, and CrkL associate with both Tyr(P)-700 and Tyr(P)-774 in vivo and that increased phosphorylation of Tyr-700, Tyr-731, and Tyr-774 in 70Z Cbl results in increased recruitment of Crk(L) and p85 PI3K adapter proteins. Interestingly, increased tyrosine phosphorylation of 70Z is not restricted to these three residues, as increased basal and activation-induced phosphorylation was also observed in 70Z Y700F/Y731F/Y774F relative to wt Y700F/Y731F/Y774F Cbl. Our findings apparently contrast with those recently reported by Feshchenko et al. (41), who did not detect appreciable tyrosine phosphorylation of the wt Cbl Y700F/Y731F/Y774F triple mutant in response to pervanadate treatment of transiently transfected Jurkat T cells. We suggest that this difference may be due to the higher sensitivity of the recombinant vaccinia virus expression system. Taken together with the general finding that the Cbl 1–655 truncation mutant is not appreciably tyrosine-phosphorylated (Refs. 34 and 41 and data not shown), we tentatively conclude that there is at least one other tyrosine in the C-terminal (amino acids 655–906) region that shows increased basal and activation-induced phosphorylation in 70Z versus wt Cbl.

The molecular mechanisms underlying 70Z Cbl-induced transformation and NFAT/AP1 activation have not been previously identified. In theory, the 70Z Cbl oncoprotein may itself act as a positive signal transducer or, alternatively, it may inhibit a negative regulator that prevents or down-regulates an activating signal. Consistent with the former possibility, several groups have previously suggested that 70Z Cbl-induced transformation may, at least in part, be due to its increased phosphotyrosine content and association with Crk(L) adapter proteins.

**Fig. 8.** Activation of NFAT and AP1 by oncogenic 70Z Cbl requires an intact PTB domain. Jurkat TAg cells were transiently transfected with vector or the indicated Cbl expression constructs together with NFAT (A) or AP1 (B) reporter gene constructs and either left unstimulated or stimulated for 15 h in the presence of PMA (10 ng/ml) plus ionomycin (1 μg/ml). C, whole cell lysates of the experiments shown in A and B were immunoblotted with anti-Cbl (C15) Abs to verify expression of transfected Cbl proteins. D, Jurkat T cells were infected with recombinant vaccinia virus as indicated and further treated as in Fig. 1A.
proteins (1, 28–30), perhaps through regulation of Ras signaling via the C3G-Rap1-Ras pathway. Indeed, our initial results demonstrated increased basal and activation-induced association of 70Z Cbl with Crk(L) and p85 PI3K adapter proteins. However, neither increased association of Crk(L) and p85 PI3K adapter proteins with wt or 70Z Cbl detectably affects basal or anti-CD3-induced Erk1/2 activation. Consistent with these findings, Thien and Langdon (30) have been unable to detect an effect of 70Z Cbl overexpression on Erk activation in fibroblasts. As Ras activation is both necessary and sufficient to activate Erk (Ref. 39 and references therein), these findings suggest that the Cbl-Crk(L) and Cbl-p85 PI3K interactions do not affect Ras signaling. Moreover, our study clearly demonstrates that the 70Z Cbl oncoprotein retains its ability to activate NFAT and AP1 in the absence of Crk(L) and p85 PI3K binding, indicating that 70Z-induced NFAT/AP1 activation is not mediated through increased association with these adapter molecules.

Although we can exclude the possibility that 70Z Cbl-mediated NFAT activation is mediated through increased phosphorylation of Tyr-700, Tyr-731, and Tyr-774, our findings demonstrate increased phosphorylation of 70Z Cbl on additional tyrosine residues. Therefore, it remains possible that increased tyrosine phosphorylation of 70Z Cbl on these additional tyrosine residues contributes to its oncogenic and NFAT/AP1 activating properties. Our finding that 70Z Cbl requires its PTB domain for induction of NFAT and AP1 is consistent with the model that mutation of the Ring finger domain of 70Z Cbl activates or exposes its N-terminal PTB domain, which would allow increased recruitment to activated protein-tyrosine kinases. Although it is not known whether the reported enhanced association of 70Z Cbl with the epidermal growth factor and platelet-derived growth factor receptor tyrosine kinases (27, 28) depends on its PTB domain, introduction of the G306E mutation into c/v-Cbl abrogates its association with activated (receptor) tyrosine kinases in vitro as well as its transforming activity in vitro (4, 5, 27, 28). Indeed, our findings also demonstrate that the G306E mutation blocks increased tyrosine phosphorylation of 70Z Cbl, suggesting that increased tyrosine phosphorylation of 70Z Cbl results from increased or prolonged recruitment to activated tyrosine kinases via its PTB domain. Considering this model, however, it seems paradoxical that the PTB domain of Cbl associates with phosphotyrosine residue 292 of ZAP70 both in vitro and in vivo (4, 5), as mutation of this tyrosine residue disrupts interaction with the Cbl PTB domain (5) yet up-regulates NFAT activation in unstimulated Jurkat T cells (42).

As discussed above, 70Z Cbl-induced transformation and NFAT/AP1 activation may also result from inhibition of a negative regulator that prevents or down-regulates an activating signal. As studies in D. melanogaster (11), C. elegans (12), and mammalian RBL 2H3 mast cells (26) indicate that Cbl functions as an evolutionary conserved negative regulator of protein-tyrosine kinases, it is possible that 70Z Cbl acts as a dominant negative by blocking the negative regulatory role of endogenous Cbl on protein-tyrosine kinase signaling pathways. In this model, the increased tyrosine phosphorylation of 70Z Cbl, which depends on its PTB domain, may be the consequence of increased recruitment to an activated tyrosine kinase via its PTB domain and/or the inability of endogenous Cbl to inhibit or down-regulate activated protein-tyrosine kinases in the presence of the 70Z Cbl PTB domain. Importantly, competitive inhibition of endogenous Cbl binding to the ZAP70 phosphotyrosine residue 292 by the 70Z Cbl PTB domain is consistent with a negative regulatory role for the interaction of endogenous Cbl with the ZAP70 Tyr-292 residue (4, 5, 42). Whether wt or 70Z Cbl interacts with ZAP70 following T cell receptor activation and whether such an interaction plays any biologically significant role in T cell receptor signal transduction remains to be determined. If this model is correct, then it remains to be determined why 70Z Cbl, but not v-Cbl, is able to activate NFAT in Jurkat T cells and up-regulate epidermal growth factor receptor kinase activity in fibroblasts.

In summary, we have determined the molecular basis for NFAT and AP1 induction by oncogenic 70Z Cbl in unstimulated Jurkat T cells. Our results demonstrate that NFAT/AP1 activation by 70Z Cbl is not mediated through increased interaction with Crk(L) and p85 PI3K adapter proteins but instead depends on an intact PTB domain. These findings are most consistent with a dominant negative action of the 70Z Cbl PTB domain on the negative regulatory role of endogenous Cbl on protein-tyrosine kinases.

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