Regulation of Ubiquitin Protein Ligase Activity in c-Cbl by Phosphorylation-induced Conformational Change and Constitutive Activation by Tyrosine to Glutamate Point Mutations*‡

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The c-Cbl proto-oncogene was first discovered as the cellular homologue of v-Cbl, a viral transforming gene from the Cas NS-1 murine retrovirus, which causes pre-B cell lymphomas and myelogenous leukemias in mice (1). The transforming gene v-Cbl is a truncation mutant of c-Cbl, which itself does not transform cells (2). Human c-Cbl encodes a widely expressed cytosolic protein of 906 amino acids, which is a prominent substrate of a variety of tyrosine kinases and undergoes binding interactions with a large number of intracellular signaling molecules (3, reviewed in Refs. 4–8). Two other related genes exist in mammals, Cbl-b (9) and Cbl-3 (10), and Cbl homologues have been identified in Caenorhabditis elegans (11) and Drosophila (12).

The amino terminus of c-Cbl contains a conserved functional domain that binds phosphotyrosine, composed of a four-helix bundle, a Ca$^{2+}$-binding EF hand domain, and a variant SH2 domain (13). These structural components together comprise a functional unit that has been called the TKB domain (for tyrosine-kinase binding). A short helical linker region connects the TKB domain to a RING finger domain that contains two bound zinc ions. The TKB, linker helix, and RING domains are well conserved among all Cbl family members.

Important clues regarding Cbl function have come from genetic studies in C. elegans and Drosophila, in which it was found that Cbl family members act to negatively regulate receptor tyrosine kinases (11, 12). Subsequent studies in mammalian cells have confirmed this negative regulatory role of Cbl, and a mechanism for the down-regulation of receptor tyrosine kinases by Cbl has been provided by the discovery of ubiquitin protein ligase activity in c-Cbl, mediated by the RING finger domain (14–19).

Ubiquitin protein ligases (abbreviated as E3s) are part of a multienzyme system for conjugating ubiquitin to substrate proteins (reviewed in Refs. 20–22). The first step in this process involves ATP-dependent formation of a thioester between the C terminus of ubiquitin and the active site cystine of ubiquitin-activating enzyme (E1). A second thioester intermediate is subsequently formed between ubiquitin and one of several ubiquitin-conjugating enzymes (abbreviated as E2s, or ubc). In the final step of the process, E2s act in concert with ubiquitin protein ligases (E3s) to form an isopeptide bond between the carboxyl terminus of ubiquitin and a free amino group on the substrate protein. In most cases, ubiquitin conjugation results in multiubiquitin chains that target the substrate for degradation by proteasomes or, in some cases, lysosomes. In a few cases, conjugation of single ubiquitin moieties can produce a relatively stable post-translational modification of certain substrate proteins.

Two major families of E3s are known, HECT proteins (homology to E6-associated protein carboxyl terminus) and RING finger-containing proteins. Although not all proteins that contain a RING domain are E3s, a large and growing number of RING proteins have been found to have ubiquitin ligase activity. The RING domain is important for binding E2s, and a recent crystal structure of a complex of an amino-terminal

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fragment of c-Cbl and the E2, Ubch7 has provided precise details of that interaction (23). The E3 activity of c-Cbl has been reported to be regulated by tyrosine phosphorylation, and a mutational study was done by Levkovitz et al. (19) to try to define the key tyrosine residues in c-Cbl responsible for that activation. In that study, a series of tyrosine to phenylalanine point mutants in c-Cbl were transfected into Chinese hamster ovary cells along with the EGF receptor, and the ability of EGF to induce ubiquitination of EGRF was measured. Wild type c-Cbl and Y to F point mutants at positions 92, 274, 291, 307, 337, and 368 were all able to enhance EGRF ubiquitination under these conditions, whereas Y371F was inactive. The authors concluded that Tyr-371 was the key site for phosphorylation-induced activation of the ubiquitin ligase activity of c-Cbl. However, this interpretation was subsequently called into question by the structural studies of Zheng et al. (23), who stated that this tyrosine residue was not solvent-accessible, and instead was internally hydrogen-bonded to threonine 227. These authors implied that tyrosine to phenylalanine mutation at Tyr-371 would disrupt the structure of the enzyme, rather than merely removing a phosphorylation site. These two models for the function of Tyr-371 would appear to be mutually exclusive.

In the studies reported here, we further investigate the mechanism of phosphorylation-induced activation of the ubiquitin ligase activity of c-Cbl. As part of this work, we have attempted to resolve the controversy over which tyrosines are required for activation by generating gain-of-function mutations in c-Cbl to try to avoid the problems inherent in loss-of-function studies.

**EXPERIMENTAL PROCEDURES**

**Construction of GST-Cbl Fusion Proteins and Cbl Point Mutants—**

All GST-Cbl fusion proteins were made from corresponding pGEX-Cbl plasmids based on the vector pGEX-4T-1 (Amersham Biosciences). Inserts were constructed by PCR using Pfu polymerase (Stratagene, La Jolla, CA), a cDNA clone of human c-Cbl provided by W. Langdon (University of Western Australia, Perth, Australia) as template, and PCR primers containing compatible restriction sites. pGEX-Cbl-RING contains an insert encoding amino acids 358–447 of c-Cbl preceded by an EcoRI site and followed by a stop codon and an NotI site. pGEX-Cbl 1–480 contains a BamHI site immediately preceding sequences encoding amino acids 1–480 of c-Cbl and followed by a stop codon and an NotI site. pGEX-Cbl 1–480 was cloned into the pGEX-2TK expression vector (GlaxoSmithKline, Research Triangle Park, NC) that contains a T7 promoter followed by a stop codon and an NotI site. pGEX-Cbl 1–480 was verified by sequencing. GST fusion proteins were produced in *Escherichia coli* strains DH5α or BL21 containing the appropriate pGEX plasmid as follows. 500-ml cultures were grown to log phase (A600 of 0.6–0.7) and induced by addition of isopropyl-1-thio-β-D-galactopyranoside to 0.1 mM final. After 3–4 h of induction, cells were harvested, rinsed with 50 mA NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and frozen at −80 °C. Cell pellets were later thawed and resuspended in 10–15 ml of 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 1 µg/ml each of leupeptin, pepstatin, bestatin, and E64. Cells were lysed by the addition of 100 µg/ml lysozyme and 1% Triton X-100 or 1% Triton (Roche Applied Science) for 15–30 min followed by sonication. Lysates were cleared by centrifugation for 15 min at 25,000 × g and then incubated with 0.5–1.0 ml of a 50% slurry of glutathione-Sepharose beads for 30 min to 1 h at 23 °C or 2–15 h at 4 °C with rotation. The beads were washed 3 × 10 ml with the above buffer containing 0.1% Triton and then placed in a column and eluted overnight at 4 °C with 20 mM reduced glutathione, 100 mM Tris, pH 8.0, 50 mM NaCl, 0.01% Triton X-100, 1 mM EDTA, and then again the same buffer containing 50% glycerol and 0.01% Triton. Dialyzed proteins were cleared of insoluble material by centrifugation at 13,000 × g for 4 °C for 15 min and stored at −20 °C. Protein concentrations were measured by the Bio-Rad protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. Tyrosine-phosphorylated proteins were prepared in the *E. coli* strain TKX1 or TKB1 induced as described by the manufacturer (Stratagene) and lysed as described above except that the lysis buffer also contained 50 mM NaF and 1 mM sodium orthovanadate and lacked DTT. GST-Cbl-b 1–480 and the corresponding Y363E mutant was produced by similar methods from a human cDNA clone provided by Stanley Lipkowitz, NCI, National Institutes of Health, Bethesda, MD.

**In Vitro Ubiquitination Assay—**

GST-ubiquitin was produced in *E. coli* using a pGEX-2TK-based expression plasmid obtained from Mark Hochstrasser, Yale University, New Haven, CT) and purified by gel filtration. GST-ubiquitin was purified as described for the GST-Cbl fusion proteins above. Purified GST-ubiquitin was 32P-labeled in HMK buffer (100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 12 mM MgCl2, 1 mM DTT) containing 0.8 µCi/µl [γ-32P]ATP (105 Ci/µl ATP was obtained from PerkinElmer Life Sciences) and 0.3 unit/µl of bovine heart kinase (Sigma #P-2645) for 30 min at 12 °C, then inactivated by heating at 100 °C for 30 s. The mixture was quenched by addition of 10 mM sodium phosphate, 10 mM sodium pyrophosphate, 10 mM EDTA, 1 mg/ml bovine serum albumin, pH 8.0. Glutathione-agarose was then added, and the mixture was rotated at 4 °C for 30 min to 1 h. The beads were washed five times with PBS containing 0.1% Thesitol and then treated with thrombin in the same buffer for 2 h at room temperature or overnight at 4 °C with rotation. Following thrombin cleavage, PMSF was added to 2 mM final, and the beads were spun and washed three times with PBS to recover the [32P]ubiquitin in the supernatant. Wheat E1 and human Ubch5b were expressed in *E. coli* from the plasmids pETU1A (Rick Vierstra, University of Wisconsin, Madison, WI, obtained from Mark Hochstrasser, Yale University, New Haven, CT) and pETU1B (Alan Weissman, NCI, National Institutes of Health, Bethesda, MD), and purified by covalent affinity chromatography on ubiquitin agarose as described (24). In *vitro* ubiquitination reactions were performed in 20 µl of total volume containing 185 ng of E1, 1 µg of Ubch5b, and 2 µg of GST-Cbl fusion proteins in a buffer containing 50 mM KCl, 20 mM HEPES, pH 7.4, 5 mM MgCl2, 1 mM DTT, 1 mM ATP, and an ATP-regenerating system consisting of 10 mM creatine phosphate, 3.5 units/ml creatine phosphokinase, and 0.6 unit/ml of inorganic pyrophosphatase. [32P]Ubiquitin was prepared to specific activities ranging from 3.5 × 106 to 4.6 × 106 cpm/µg, and 100,000–500,000 cpm was added per reaction. Reactions were set up on ice and then transferred to 30 °C for various times; time points were quenched by adding an equal volume of 2× SDS sample buffer followed by boiling.

**Protease Digestions—**

GST fusion proteins were first cleared with thrombin, treated with PMSF and glutathione-Sepharose to inactivate thrombin and remove GST, and then dialyzed and concentrated into 50 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 50% glycerol. The resulting Cbl 1–480 proteins were stored at −20 °C. For digestions, proteins were adjusted to 0.2 mg/ml in buffer lacking glycerol and digested with protease at 10 µg/ml at 37 °C. Proteinase K was obtained from Sigma, and digests were performed in the above buffer lacking glycerol.

**Trypsin digestions** were performed similarly using Sequencing Grade trypsin from Promega (Madison, WI) and then dialyzed against: 185 ng E1, 100 mM NaCl, 100 mM Tris-HCl, pH 7.9, 1 mM CaCl2, 1 mM DTT. Aliquots were removed at the indicated time points, treated with PMSF, and then boiled in SDS sample buffer. Samples were analyzed by SDS-PAGE on 12% gels followed by Coomassie staining.

**Identification of phosphorylation sites** were 50 µg of P-Cbl 1–480 was adjusted to 1% SDS and 5 mM DTT and boiled for 5 min. After cooling, 10 µl of 100 µg/ml insulin, and the sample was incubated 30 min at room temperature in the dark. The sample was then diluted 10-fold into 1% Trition, 100 mM NaCl, 100 mM Tris-HCl, pH 7.9, 1 mM CaCl2, and digested with 2.5 µg of trypsin for 7 h at 37 °C followed by 15 h at room temperature. PMSF was then added to a final concentration of 2 mM followed by 10 µl of a 50% slurry of agarose beads containing immobilized monoclonal anti-phosphotyrosine antibody (4G10, Upstate Cell Signaling Solutions, catalog number 16-101). The sample was rotated at room temperature for 3 h, and the beads were then spun out and washed three times with PBS and three times with H2O. All supernatant was removed, and the beads were eluted with 0.1% trifluoroacetic acid in acetonitrile, for analysis by MALDI-TOF mass spectrometry. Expected mass differences were measured using the program ProteinProspector.

**Cell Line Preparation and EGRF Binding Assay—**

The human mammary epithelial cell line MCF12A was obtained from the University of Colorado Cancer Center Tissue Culture Core Facility and cultured in 50:50 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 supplemented with 5% horse serum, 1 mM l-glutamine, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 0.1 µg/ml cholera toxin, 20

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The TKB and/or linker helix domains of c-Cbl regulate the ubiquitin ligase activity of the RING domain in a phosphorylation-dependent manner. In vitro ubiquitination reactions employing [32P]-labeled ubiquitin were performed as described under “Experimental Procedures” using purified proteins produced in E. coli. All reactions contained E1, UbcH5b as E2, an ATP-regenerating system, and various GST-Cbl fusion proteins as E3 as indicated at the top of the figure. Lanes 1–6 labeled as “GST” GST alone; lanes 7–12 labeled as “GST-Cbl-RING,” a GST fusion with amino acids 358–447 of c-Cbl (the RING domain and a few flanking amino acids); lanes 13–18 labeled as “GST-Cbl 1–480,” a GST fusion of the entire amino-terminal half of c-Cbl (including TKB domain, linker helix, and RING domains); and lanes 19–24 indicated “P-GST-Cbl 1–480,” the same construct as lanes 13–18 but produced under conditions that phosphorylated tyrosine residues. Reactions were assembled on ice then transferred to 30 °C and incubated for various times (0–40 min) as shown at the top of the figure. Reactions were terminated by the addition of SDS sample buffer followed by boiling. Samples were analyzed by SDS-PAGE on 8% gels, followed by autoradiography. Lane numbers are indicated at the bottom of the figure, and the positions of molecular weight markers are indicated on the side.

Figure 1: The TKB and/or linker helix domains of c-Cbl regulate the ubiquitin ligase activity of the RING domain in a phosphorylation-dependent manner. The carboxyl-terminal half of c-Cbl and Cbl-b is dispensable for EGF-induced ubiquitination of EGFR and PDGFR; fragments containing approximately the first 480 amino acids are sufficient, as is Cbl-3, which is only 474 amino acids in length (10, 18, 19). To investigate the regulation of the E3 activity of c-Cbl, we have therefore focused our investigations on this region of the molecule. We first set out to establish an in vitro ubiquitination system, using only purified components expressed in E. coli. The system contains [32P]-labeled ubiquitin, E1, UbcH5b as E2, GST-c-Cbl fusion proteins as E3s, and an ATP-regenerating system. We first examined the behavior of a GST fusion construct containing amino acids 358–447 of c-Cbl, essentially the RING finger domain and a few flanking amino acids (GST-Cbl-RING). A similar construct has been reported to demonstrate ubiquitin ligase activity in vitro (18). Similar to previous reports, we find that GST-Cbl-RING is readily auto-ubiquitinated (Fig. 1, lanes 1–12). Auto-ubiquitination of E3s is commonly observed and provides a convenient assay for ubiquitin ligase activity in vitro. No E3 activity was observed with GST alone (Fig. 1, lanes 1–6) or in the absence of ATP, E1, or E2 (data not shown). In contrast to GST-Cbl-RING, a GST fusion protein containing the entire amino-terminal half of c-Cbl (GST-Cbl 1–480) showed much less activity in this assay, suggesting that the intrinsic E3 activity of the RING domain may be inhibited by interactions from the TKB domain and/or linker helix region present in this larger construct (Fig. 1, compare lanes 13–18 with 7–12; note also that ubiquitinated GST-Cbl-RING species exhibit increased mobility due to the smaller size of this construct).

To test whether this putative inhibition might be modulated by tyrosine phosphorylation, we next prepared a tyrosine-phosphorylated form of GST-Cbl 1–480, (P-GST-Cbl 1–480) using the bacterial strain TKX1, which contains an inducible elk1 tyrosine kinase. The protein purified from this strain was confirmed to contain phosphotyrosine using the phosphotyrosine-specific monoclonal antibody 4G10 (data not shown). It should be noted that we have no data at present indicating whether or not the pattern of tyrosine phosphorylation in our bacterially produced protein accurately reflects the tyrosine phosphorylation occurring in mammalian cells. However, we do find that bacterially produced P-GST-Cbl 1–480 has dramatically enhanced ubiquitin ligase activity over the unphosphorylated protein (Fig. 1, compare lanes 19–24 with 13–18; note also that E3 activity is evident even in the zero time point for P-GST-Cbl 1–480, in which the sample remained on ice rather than incubating at 30 °C). To rule out any possible effects of the GST portion of these fusion proteins, we prepared Cbl 1–480 and P-Cbl 1–480 free from GST by treating the fusion proteins with thrombin. Cbl 1–480 and P-Cbl 1–480 prepared in this way retained the ubiquitin ligase activities of the parent GST fusion proteins (data not shown). Taken together, these data argue that the TKB domain and/or linker helix region of c-Cbl regulate the intrinsic E3 activity of the RING domain in a phosphorylation-dependent manner.

Analyzing the Phosphorylation-induced E3 Activity of c-Cbl Using Tyrosine to Phenylalanine Point Mutations: Tyr-268 Is Not Required for Activation—Previous investigators have examined the question of which tyrosine residues in c-Cbl are critical for activation of its ubiquitin ligase activity, however the question remains controversial. Analysis is complicated by
the large number of residues to be considered; c-Cbl contains 22 tyrosine residues. However, only six of these are completely conserved between the five known Cbl family members, corresponding to residues 268, 274, 291, 307, 337, and 371 of c-Cbl. Two additional tyrosine residues are conserved in four of the five Cbl family members (corresponding to residues 92 and 368 of c-Cbl). Levkowitz et al. (19) approached the question by generating a series of tyrosine to phenylalanine point mutants in c-Cbl; they found that Tyr to Phe point mutations at sites 92, 274, 291, 307, 337, and 368 retained activity in their assay, whereas a Y371F mutant was inactive. However, as discussed earlier, the interpretation of this result has been controversial, with Zheng et al. (23) arguing from their structural studies that a Y371F mutation would disrupt the structure of c-Cbl, rather than merely removing a regulatory phosphorylation site (23). If one accepts this argument for the moment, what other conclusions can be drawn from the data of Levkowitz et al.? Because none of the other Tyr to Phe point mutations tested abolished E3 activity, two possibilities present themselves. The first is that the key tyrosine residue required for phosphorylation-induced E3 activation was not among those tested. The second possibility is that more than one tyrosine residue in c-Cbl can activate E3 activity through phosphorylation, i.e. no single tyrosine residue is absolutely required (except for the proposed structural role played by Tyr-371). Of course it is also possible that one of the non-conserved tyrosines is the key phosphorylation site, but we consider this possibility less likely. If more than one tyrosine is involved, the number of possible permutations grows large and makes analysis by multiple Tyr to Phe mutation unattractive. Attempting to construct inactive mutants through Tyr to Phe mutation also suffers from the earlier problem that more than one interpretation is possible. We therefore chose to try a different approach; we constructed a series of tyrosine to glutamate point mutants in c-Cbl, in an attempt to partially mimic the effect of tyrosine phosphorylation by introducing fixed negative charge. Although glutamate is not expected to closely mimic phosphotyrosine, the effect we are looking for is a gain of function, rather than a loss of function, and thus likely to be informative, if observed. In Fig. 3, we analyze the E3 activity of a series of Tyr to Glu point mutations in c-Cbl, in an attempt to partially mimic the effect of tyrosine phosphorylation by introducing fixed negative charge. Glutamate mutants did indeed show increased constitutive E3 activity in this assay. In particular, Tyr to Glu mutations at positions 307, 337, and 371 show strongly increased activity over unphosphorylated wild type (Fig. 3, compare lanes 8–10 with lane 3), and Y274E also appears to have slightly increased activity
Regulation of E3 Activity in c-Cbl

FIG. 3. Ubiquitin ligase activity of tyrosine to glutamate point mutants of c-Cbl. In vitro ubiquitination reactions were performed as described previously for 1 h at 30 °C using various GST fusion proteins as E3, as indicated at the top of the figure. Lane numbers are indicated at the bottom. Lane 1 labeled as “GST,” GST lacking any c-Cbl residues; lane 2 labeled as “RING,” a GST fusion with amino acids 358–447 of c-Cbl (the RING domain and a few flanking amino acids); lane 3 labeled as “1–480,” a GST fusion of the amino-terminal half of wild type c-Cbl (amino acids 1–480, including TKB domain, linker helix, and RING domains); lane 4 labeled as “P 1–480,” wild type GST-Cbl 1–480 with phosphorylated tyrosine residues; and lanes 5–10, tyrosine to glutamate point mutations of GST-Cbl 1–480 as indicated. Shown is an autoradiograph of a 7% gel; the positions of molecular weight markers are indicated at the side of the figure.

(Fig. 3, compare lane 6 to lane 3). Y268E and Y291E are not active above wild type levels in this assay (Fig. 3, compare lanes 5 and 7 with lane 3). Quantitation of the levels of auto-ubiquitination observed in this assay indicates that the strongly activated 307, 337, and 371 mutants incorporated 30–43% as much [32P]ubiquitin as phosphorylated wild type P-GST-Cbl 1–480.

Y371E Mutation in c-Cbl Enhances Binding to Activated EGFR—Two possibilities could explain the activation of ubiquitin ligase activity observed in the Y274E, Y307E, Y337E, and Y371E mutants. The first is that, as hoped, tyrosine to glutamate mutations are partially mimicking the effects of tyrosine phosphorylation at these sites, and therefore some or all of these sites might represent physiological phosphorylation sites involved in the activation of the E3 activity of c-Cbl. However, an alternative explanation might be that these mutations simply disrupt the structure of the TKB and/or linker helix domains, and this disruption removes the negative regulation exerted by these domains on the RING E3 activity. Indeed, the crystal structures of c-Cbl fragments obtained by Meng et al. and Zheng et al. indicate that conserved tyrosines 274, 307, and 337, are all involved in forming the binding pocket of the TKB domain (13, 23). In addition, as described earlier, Zheng et al. (23) have argued that Tyr-371 makes important internal structural contacts. These are precisely the tyrosine residues at which glutamate substitution increases E3 activity. Therefore, to assess the integrity of the TKB domain, we assayed the ability of these constructs to bind to the EGFR receptor.

In Fig. 4, equal amounts of the indicated GST fusion proteins were added to cell extracts prepared from MCF12A cells, a human mammary epithelial cell line containing abundant EGFR receptors. Cell extracts were prepared from both quiescent (Fig. 4B) and EGFR-stimulated (Fig. 4A) cells. Control experiments indicated that EGFR in the EGFR-stimulated cell extracts was phosphorylated on tyrosine residues as expected and that EGFR in extracts of quiescent cells lacked detectable phosphate-tyrosine (not shown). GST fusion proteins, and any cellular proteins bound to them, were then recovered by binding to glutathione-agarose. After washing, bound proteins were analyzed by SDS-PAGE followed by Western blotting with anti-EGFR antibody. In Fig. 4, it can be seen that GST alone does not bind EGFR, nor does GST-Cbl-RING, which lacks the TKB domain (Fig. 4A and B, lanes 2 and 3). However, as expected, wild type GST-Cbl 1–480 does bind EGFR, but only the activated, tyrosine-phosphorylated form from EGFR-stimulated cells (compare lanes 4 in Fig. 4, A and B). Furthermore, our bacterially prepared tyrosine-phosphorylated P-GST-Cbl 1–480 also binds EGFR in an activation-specific manner (Fig. 4A, B and B, lanes 5). Of the six tyrosine to glutamate point mutants in c-Cbl, only Y371E binds to activated EGFR (Fig. 4A, compare lane 11 with lanes 6–10). Consistent with the structural studies mentioned above, activated EGFR no longer binds to the Tyr to Glu mutants at positions 274, 307, and 337, which lie in the TKB binding pocket (Fig. 4A, lanes 7, 9, and 10). It is notable that another Cbl mutant known to disrupt EGFR binding, G306E, is adjacent to one of these sites. We also do not see significant EGFR binding to Y268E or Y291E (Fig. 4A, lanes 6 and 8).

The behavior of the Y371E mutant in these studies is notable. Not only does Y371E retain the ability to bind to the activated EGFR receptor, it actually appears enhanced over wild type (Fig. 4A, compare lane 11 with lane 4). In this regard it is worth noting that we also find reproducibly greater binding of EGFR to phosphorylated wild type GST-Cbl 1–480 than unphosphorylated GST-Cbl 1–480 (Fig. 4A, compare lanes 4 and 5), although this enhanced binding is not as pronounced as that seen in the Y371E mutant. These data indicate that the TKB...
domain is not grossly disrupted in the Y371E mutant and argue instead that glutamate mutation at this site may partially mimic the effect of tyrosine phosphorylation.

Tyrosine Phosphorylation of c-Cbl 1–480 Induces a Conformational Change—The data from our Y371E mutant support the contention of Levkowitz et al. that Tyr-371 is a key site for regulation of the ubiquitin ligase activity of c-Cbl. How can this conclusion be reconciled with Zheng et al.’s structural studies? Zheng et al. found in their crystal structure that Tyr-371 is hydrogen-bonded to threonine 227 in an internal location inaccessible to solvent (23). They suggested that phosphorylation of Tyr-371 would have to involve a significant structural rearrangement of the linker-TKB and linker-E2 interfaces. We therefore set out to look for evidence of such structural rearrangement.

If tyrosine phosphorylation of c-Cbl significantly alters its conformation, this may be reflected in altered susceptibility to proteases. To examine this possibility directly we performed protease digestion studies on purified Cbl 1–480 and P-Cbl 1–480 free from GST. Our purified Cbl 1–480 and P-Cbl 1–480 preparations show variable amounts of slightly smaller species, which likely represents slight proteolytic cleavage of the carboxyl terminus occurring during purification from the bacterial lysates (Fig. 5, A and B, lanes 1 and 2). We prepared equal concentrations of phosphorylated and unphosphorylated c-Cbl 1–480 and subjected these proteins to protease digestions in parallel, at a 20:1 ratio of substrate protein to protease. At various time points, aliquots of the reactions were removed and analyzed by SDS-PAGE and Coomassie staining. Fig. 5A shows the results of digestion with proteinase K, a protease with broad cleavage specificity. Panel B shows the results of a digest with trypsin, which has a more restricted cleavage specificity.

In both cases, the tyrosine-phosphorylated form of Cbl 1–480 is digested more readily than the unphosphorylated protein. Furthermore, in the trypsin digests, a different pattern of proteolytic intermediates is observed (Fig. 5B, lanes 3–10, compare even and odd pairs of lanes). These data indicate that tyrosine phosphorylation of c-Cbl causes the protein to adopt a different conformation, possibly a more open conformation, more accessible to added proteases.

The Y371E Point Mutant of c-Cbl Has an Altered Conformation—The protease susceptibility studies above provide direct evidence that tyrosine phosphorylation of c-Cbl alters its conformation. We have also presented evidence above that mutation of tyrosine 371 to glutamate may partially mimic the effects of tyrosine phosphorylation. We therefore asked whether the Y371E mutant of c-Cbl has an altered conformation from wild type, as evidenced by protease susceptibility. Fig. 6 shows that indeed the Y371E mutant of c-Cbl is digested differently by proteases than unphosphorylated wild type. In this experiment equal concentrations of wild type and Y371E

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**Fig. 5.** Tyrosine phosphorylation of c-Cbl alters its protease susceptibility. c-Cbl 1–480 protein was produced in E. coli in both unphosphorylated and tyrosine phosphorylated forms and cleaved from GST as described. Equal amounts of purified proteins were then digested with either proteinase K (shown in A) or trypsin (shown in B) at 37 °C, and aliquots were removed at various times during the digestion. Reactions were stopped by the addition of PMSF followed by boiling in SDS sample buffer. Samples were analyzed by SDS-PAGE on 12% gels followed by Coomassie staining. At the top of each panel the digestion times in minutes or hours are indicated. Unphosphorylated c-Cbl 1–480 is present in the odd-numbered lanes, and tyrosine-phosphorylated c-Cbl 1–480 is contained in the even-numbered lanes (marked with "P" at the top of the panels). Lane numbers are shown at the bottom of each panel, and the position of molecular weight markers are shown on the side.

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**Fig. 6.** Tyrosine to glutamate mutation of Tyr-371 alters the protease sensitivity of c-Cbl 1–480 in a manner similar to tyrosine phosphorylation. Shown are Coomassie-stained gels of protease digestions, similar to Fig. 5. Lane numbers are indicated at the bottom of each panel, and the positions of molecular weight markers are indicated at the side. Digestion times in minutes or hours are indicated at the top of each panel. In A and B, protease digestions of c-Cbl 1–480 wild type (odd-numbered lanes) or Y371E mutant (even-numbered lanes) were performed as in Fig. 5. Both proteins were unphosphorylated. Samples in A were digested with proteinase K; in B, trypsin was employed. In C, unphosphorylated Y371E mutant c-Cbl 1–480 (even-numbered lanes) is compared with tyrosine-phosphorylated wild type c-Cbl 1–480 (odd-numbered lanes) in a trypsin digest.
A Y363E mutant in Cbl-b behaves similarly to the Y371E mutant of c-Cbl. A, the Y363E mutant of Cbl-b has constitutively activated ubiquitin ligase activity. GST fusion proteins were employed in ubiquitination reactions with 32P-labeled ubiquitin as described previously. Shown is an autoradiogram of the reaction products analyzed on SDS-PAGE. Lane numbers are shown at the bottom of the panel, and the position of molecular weight markers are indicated at the side. GST fusion proteins assayed for E3 activity are indicated at the top and are as follows: lane 1 labeled as “P 1–480,” tyrosine-phosphorylated GST-Cbl 1–480; lane 2 labeled as “I–480,” unphosphorylated GST-Cbl 1–480; lane 3 labeled as “Y371E,” the Y371E mutant of GST-Cbl 1–480; lane 4 labeled as “GST,” GST lacking additional sequences; lane 5 labeled as “Cbl-b 1–480,” a GST fusion of amino acids 1–480 of Cbl-b; and lane 6 labeled as “Cbl-b Y363E,” the indicated point mutant of Cbl-b. B, the Y363E mutant of Cbl-b retains EGFR binding ability. Shown is a GST pull-down experiment similar to Fig. 4. The GST fusion proteins indicated at the top of the panel (notations are the same as in A) were incubated with cell lysates of EGF-stimulated MCF12A cells as described, and bound proteins were analyzed by EGFR immunoblot.

Fig. 7. A Y363E mutant in Cbl-b behaves similarly to the Y371E mutant of c-Cbl. A, the Y363E mutant of Cbl-b has constitutively activated ubiquitin ligase activity. GST fusion proteins were employed in ubiquitination reactions with 32P-labeled ubiquitin as described previously. Shown is an autoradiogram of the reaction products analyzed on SDS-PAGE. Lane numbers are shown at the bottom of the panel, and the position of molecular weight markers are indicated at the side. GST fusion proteins assayed for E3 activity are indicated at the top and are as follows: lane 1 labeled as “P 1–480,” tyrosine-phosphorylated GST-Cbl 1–480; lane 2 labeled as “I–480,” unphosphorylated GST-Cbl 1–480; lane 3 labeled as “Y371E,” the Y371E mutant of GST-Cbl 1–480; lane 4 labeled as “GST,” GST lacking additional sequences; lane 5 labeled as “Cbl-b 1–480,” a GST fusion of amino acids 1–480 of Cbl-b; and lane 6 labeled as “Cbl-b Y363E,” the indicated point mutant of Cbl-b. B, the Y363E mutant of Cbl-b retains EGFR binding ability. Shown is a GST pull-down experiment similar to Fig. 4. The GST fusion proteins indicated at the top of the panel (notations are the same as in A) were incubated with cell lysates of EGF-stimulated MCF12A cells as described, and bound proteins were analyzed by EGFR immunoblot.

mutant c-Cbl 1–480 protein, cleaved from GST, were incubated with either proteinase K (Fig. 6A) or trypsin (Fig. 6B), as in the previous experiment, and aliquots were removed over time. As noted in the previous experiment, some heterogeneity is evident in the starting material; nonetheless, it is evident that the two proteins differ in both the rate of digestion and the pattern of proteolytic intermediates formed. Similar to the case of phosphorylated c-Cbl, Y371E is initially degraded more rapidly than unphosphorylated wild type c-Cbl. Interestingly, at later time points, a fragment of the Y371E mutant appears to have increased stability over wild type (Fig. 6, for example compare lanes 15 and 16 in panel A, or lanes 13 and 14 in panel B). In the tryptic digestion of Y371E, a discrete band is prominent with a mobility just above the 37-kDa marker. A similar discrete tryptic fragment was prominent in the digestion of tyrosine-phosphorylated wild type c-Cbl (see Fig. 5, lanes 4, 6, 8, and 10). The molecular weight markers on that gel might suggest a fragment of different size, however the markers on the gels in Fig. 5 were commercial pre-stained markers that exhibited quite broad bands on the gel, and thus they provide only a rough guide to the true electrophoretic mobilities. The markers used in Fig. 6 were much sharper and more accurate. For example, c-Cbl 1–480 has a predicted molecular mass of ~54 kDa, more in agreement with the electrophoretic markers used in Fig. 6. To more accurately determine if the tryptic digestion of phosphorylated c-Cbl 1–480 and Y371E mutant c-Cbl shared any similarities in rate or pattern of digestion, the two proteins were digested at equal concentrations under identical conditions and run side by side, as is shown in Fig. 6C. Phosphorylated wild type protein is present in the odd-numbered lanes, and mutant unphosphorylated protein is shown in the even-numbered lanes. It can be seen that a discrete tryptic fragment of ~38 kDa, relatively resistant to proteolytic cleavage, is prominent in both digests. The digestion pattern of the Y371E mutant more closely resembles that of tyrosine-phosphorylated c-Cbl rather than the unphosphorylated form. This is evidence that the Y371E mutant has an altered conformation from wild type and provides further evidence that the Y371E mutant may be a model for tyrosine-phosphorylated c-Cbl.

Examination of primary sequence and tertiary crystal structures of c-Cbl for tryptic cleavage sites suggests a possible identity for the tryptic fragment of ~38 kDa resistant to proteolytic cleavage in the Y371E mutant and tyrosine-phosphorylated wild type. The structures forming the TKB domain (four helix bundle, EF-hand domain, and divergent SH2 domain) pack tightly together and are likely to form a relatively resistant core. Because glutamic acid substitution at position 371 stabilizes the tryptic fragment, this suggests that the linker helix is also part of this resistant fragment. A tryptic peptide containing residues 54–382 of c-Cbl appears most likely. Such a fragment has a predicted size of 38,329 and would include the entire tightly folded TKB domain as well as the linker helix, while removing less tightly structured residues at the amino terminus, and essentially the entire RING finger domain.

It may be noted that the tryptic digest pattern of tyrosine-phosphorylated c-Cbl 1–480 in Fig. 6C differs somewhat from that in Fig. 5B. This may be due to differences in glycerol concentrations between the two digests (10% in Fig. 5B versus 20% in Fig. 6C), with the higher glycerol concentration lending greater stability to the protein.

A Y363E Mutation in Cbl-b Behaves Similarly to c-Cbl Y371E—Based on all the evidence discussed above, we believe that the TKB and/or linker helix domains of c-Cbl inhibit the ability of the RING finger domain to function as a ubiquitin ligase and that phosphorylation at tyrosine 371 causes a conformational change in the protein that removes this negative regulation, resulting in enhanced ubiquitin ligase activity. We would also argue that our Y371E mutant partially mimics the effect of tyrosine phosphorylation at this site. If this model is correct, we would predict that tyrosine to glutamate point mutation would have similar effects in other Cbl family members. To test this prediction we prepared GST fusion proteins containing the amino-terminal half of Cbl-b (GST-Cbl-b 1–480), in both wild type and mutant forms (Cbl-b Y363E; tyrosine 363 in Cbl-b is the homologue of Tyr-371 in c-Cbl). In Fig. 7A, we examine the behavior of wild type and Y363E GST-Cbl-b fusion proteins in our in vitro ubiquitination assay. We find that wild type GST-Cbl-b shows a low but evident level of auto-ubiquitination in this assay, and that the Y363E mutant has much greater activity (Fig. 7A, compares lanes 5 and 6). This behavior is very similar to that of c-Cbl (Fig. 7A,
We also tested the ability of these GST-Cbl-b proteins to bind to activated EGFR. In Fig. 7B, equal amounts of purified GST fusion proteins were incubated with cell lysates prepared from EGF-stimulated MCF12A cells. Following incubation, GST proteins, and any cellular proteins bound to them, were recovered by binding to glutathione-agarose. The presence of bound EGFR in these complexes was then assayed by Western blotting. It can be seen that both wild type and the Y363E mutant of Cbl-b bind activated EGFR in this assay, whereas GST alone does not (Fig. 7B, lanes 2–4). Similar to the findings in c-Cbl, Tyr to Glu mutation at tyrosine 363 in Cbl-b does not disrupt the function of the TKB domain and in fact may even enhance EGFR binding (Fig. 7B, compare lanes 3 and 4).

**Tyrosines 368 and 371 of c-Cbl Can Both Be Phosphorylated**—We propose that the constitutively active Y371E c-Cbl mutant partially mimics the effect of tyrosine phosphorylation at this site. To confirm this model, we next sought to identify the sites of tyrosine phosphorylation in our bacterially produced c-Cbl 1–480 protein. We electrophoresed equal amount of Cbl 1–480 and P-Cbl 1–480 on SDS-polyacrylamide gels and performed in-gel tryptic digestions followed by MALDI-TOF of the resulting peptides. Peptides containing 9 of the 14 tyrosine residues present in c-Cbl 1–480 were identified in this analysis. Peptides containing tyrosines 83, 92, 102, 114, 141, 268, 274, 291, and 337 were recovered from both samples in approximately equal amounts, however, phosphorylated forms of these peptides were not observed. We therefore believe that these tyrosine residues are not significantly phosphorylated in our bacterially produced protein. However we were unable to recover the tryptic peptide containing tyrosine 371 (which also contains tyrosine 368) under these conditions from either sample. In addition, we did not recover peptides containing tyrosines 235, 307, or 455. Peptides containing these residues may be too hydrophobic to elute from the gel under the conditions employed. We therefore pursued a different strategy to attempt to recover tyrosine-phosphorylated peptides. P-Cbl 1–480 was reduced and denatured by treatment with DTT and SDS at 95 °C. The protein was then alkylated and diluted into nonionic detergent for trypsin digestion. Phosphotyrosine-containing peptides were recovered by binding to the anti-phosphotyrosine monoclonal antibody 4G10 immobilized on agarose beads. MALDI-TOF analysis of eluate from the beads revealed two peptides with masses expected for phosphorylated tryptic fragments of c-Cbl. Both are predicted to derive from the tryptic peptide VTQEQYELYCEMGSTFQLCK from residues 361–372 of c-Cbl 1–480 protein. We electrophoresed equal amount of purified GST fusion proteins incubated with cell lysates prepared from EGF-stimulated MCF12A cells. Following incubation, GST proteins, and any cellular proteins bound to them, were recovered by binding to glutathione-agarose. The presence of bound EGFR in these complexes was then assayed by Western blotting. It can be seen that both wild type and the Y363E mutant of Cbl-b bind activated EGFR in this assay, whereas GST alone does not (Fig. 7B, lanes 2–4). Similar to the findings in c-Cbl, Tyr to Glu mutation at tyrosine 363 in Cbl-b does not disrupt the function of the TKB domain and in fact may even enhance EGFR binding (Fig. 7B, compare lanes 3 and 4).

**Phosphorylation of Tyr-371 Mutants Enhances E3 Activity**—Identification of phosphorylation at tyrosine 368 in addition to tyrosine 371 led us to ask if phosphorylation at this site has functional significance. Levkowitz et al. found that a Y368F mutant in c-Cbl was still able to ubiquitinate EGFR, indicating that phosphorylation at this site is not absolutely required for the E3 activity of Cbl. Might it contribute to activation, however? If phosphorylation of Tyr-371 alone mediates Cbl activation, we would predict that tyrosine phosphorylation of our Y371F and Y371E mutants would have no effect upon their E3 activity. However this is not what we observed; as shown in Fig. 9, tyrosine phosphorylation of both the inactive Y371F mutant and the constitutively active Y371E mutant increased their E3 activities over the unphosphorylated forms (Fig. 9, compare lanes 4 and 5, and lanes 6 and 7). When these data were quantitated by phosphorimaging and normalized to an activity of 100% for phosphorylated wild type Cbl, the Y371F mutant is found to increase from 9.5% to 39.5% upon phosphorylation, and the Y371E mutant increased from 32.9% to 79.8%. These data indicate that tyrosine 371 is not the sole site of phosphorylation responsible for activation of the ubiquitin ligase activity of Cbl.

**Phosphorylation of Tyr-368, Tyr-371 Double Mutants Does Not Alter Their E3 Activity**—The data of Fig. 9 indicate that tyrosine phosphorylation at sites other than Tyr-371 (possibly

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**FIG. 8.** Both Tyr-368 and Tyr-371 of c-Cbl are phosphorylation sites. P-Cbl 1–480 was digested with trypsin and then immunoprecipitated with anti-phosphotyrosine antibodies immobilized on agarose beads as described under "Experimental Procedures." Bound peptides were eluted and analyzed by MALDI-TOF mass spectrometry. Shown is a portion of the mass spectrum with three peaks labeled A, B, and C, and their measured masses are shown above. The mass measured for peak A is in good agreement with that expected for the tryptic peptide VTQEQYELYCEMGSTFQLCK, residues 363–382 of c-Cbl, containing one phosphate group (predicted mass 2594.07). The mass measured for peak C is in good agreement with that expected for the same tryptic peptide containing two phosphate groups (predicted mass 2674.03). Peak B corresponds to the non-phosphorylated peptide DAFQPHHSHH-HHHLSPHPGTVDK (residues 31–53 of c-Cbl with expected mass of 2665.27), apparently adsorbed non-specifically to the agarose beads.
Fig. 9. Tyr-371 mutants of c-Cbl can still be activated by phosphorylation. In vitro ubiquitination reactions were performed with 32P-labeled ubiquitin as described previously using the indicated GST fusion proteins as E3. Lane numbers are indicated at the bottom. Lane 1, GST only; lanes 2–7, GST-Cbl 1–480 fusion proteins, either wild type (“W.T.”) or Tyr-371 point mutants, as indicated. Tyrosine-phosphorylated proteins are indicated by “P,” in lanes 3, 5, and 7. The positions of molecular weight markers are indicated at the side. Shown in A is an autoradiograph of a 7% SDS-PAGE gel of the ubiquitination reaction products. Phosphorimaging quantitation of this data is shown below the lane numbers, with the data normalized to the percentage of the value of P-W.T. B, a Coomassie Blue-stained 12% polyacrylamide gel containing 2 μg of each of the GST fusion proteins used in the ubiquitination reactions in A.

Tyr-368, for example) can contribute to E3 activation in c-Cbl. Our mass spectroscopy data of Fig. 8 positively identified both Tyr-368 and Tyr-371 as phosphorylation sites. If these two residues are the only significant sites, we would predict that Tyr-368, Tyr-371 double mutants would no longer show an increase in E3 activity upon phosphorylation. We tested this prediction by constructing FF and EE double mutants at Tyr-368 and Tyr-371 and preparing GST-Cbl fusion proteins in both unphosphorylated and tyrosine-phosphorylated forms. We then measured their activities in the in vitro ubiquitination assay. We found that the Y368F/Y371F double mutant had a low basal level of E3 activity, similar to unphosphorylated wild type, and this basal level was not increased upon tyrosine phosphorylation (data not shown). The Y368E/Y371E double mutant in contrast, was constitutively activated, and in fact appeared to have somewhat greater activity than the Y371E single mutant (Fig. 10A, compare lanes 1 and 2). However, the ubiquitin ligase activity of the EE double mutant did not further increase upon phosphorylation (Fig. 10A, compare lanes 2 and 3). Control experiments confirmed that the phosphorylated forms of the FF and EE double mutants did in fact contain phosphotyrosine, as evidenced by reactivity with the 4G10 monoclonal anti-phosphotyrosine antibody, although the extent of phosphorylation was significantly reduced (appearing less than 5% of the level of the phosphorylated Y371E single mutant, data not shown). In Fig. 10C, we have assessed whether glutamate mutation at Tyr-368 affects ability to bind to activated EGFR. It can be seen that Y368E mutation, singly or in combination with Y371E, does not disrupt the ability of c-Cbl to bind activated EGFR (Fig. 10C, compare lanes 5 and 7 with lanes 3 and 6), indicating that alteration of this residue does not disrupt the TKB domain. The Y274E point mutant, which does disrupt the TKB domain, is included here as a negative control (Fig. 10C, lane 4). Because Tyr-368, Tyr-371 double mutants no longer showed phosphorylation-induced activation, we conclude that these two residues are the critical phosphorylation targets for ubiquitin ligase activation.

**DISCUSSION**

In this study we have used an in vitro ubiquitination system to explore the phosphorylation-induced activation of the ubiquitin ligase (E3) activity of c-Cbl. Activation of the E3 activity of c-Cbl by tyrosine phosphorylation has been reported by others in several systems (19, 25). We show here that the intrinsic E3 activity of the isolated RING finger domain of c-Cbl is negatively regulated by the amino-terminal TKB and/or linker helix domains when these are included in a larger construct. We also show that the activation of the ubiquitin ligase activity of c-Cbl by tyrosine phosphorylation appears to result from removal of the inhibitory effects of the amino-terminal domains. We provide direct evidence that tyrosine-phosphorylation-
ated c-Cbl adopts a different conformation than the unphosphorylated form, based on protease sensitivity studies. This phosphorylation-induced conformational change provides a mechanism to explain the regulation of E3 activity.

A debate has existed concerning the role of Tyr-371 in the activation of the E3 activity of c-Cbl, with mutational data and structural data seemingly at odds. Because loss of function mutants are always subject to multiple interpretations, we attempted here to generate activating mutations in c-Cbl, in which the negative regulation of the amino-terminal TKB and linker helix domains upon the RING was removed or reduced. Substitution of conserved tyrosine residues with glutamate was employed in the hope that introduction of fixed negative charge might, in some modest way, partially mimic the effects of tyrosine phosphorylation. At four of the six completely conserved tyrosine residues in Cbl, we found that mutation to glutamate resulted in point mutants showing increased auto-ubiquitination in our assay, indicating that altering the TKB and/or linker helix domain structure can indeed remove or reduce the negative regulatory effects exerted on the RING E3 domain. However, in vivo, Cbl proteins bind and ubiquitinate receptor tyrosine kinases, in addition to undergoing auto-ubiquitination. Thus the TKB domain is critical for biological function, and activating the E3 activity of Cbl in vivo must occur by structural changes that do not abolish TKB function. In three of the four mutants where constitutive activation was observed, the function of the TKB domain was compromised, resulting in loss of the ability to bind activated EGFR. However, mutation of tyrosine 371 to glutamate proved a notable exception. The Y371E mutant not only retains ability to bind activated EGFR, this binding appears enhanced over wild type and thus echoes the enhancement of EGFR binding observed with phosphorylation of wild type c-Cbl. We therefore suggest that the Y371E mutant may be a model for tyrosine-phosphorylated c-Cbl. We generalized this model by studies with Cbl-b and show that an homologous Cbl-b-Y363E mutant behaves similarly. To confirm our model we identified Tyr-371 as a site of phosphorylation in our bacterially produced protein and unexpectedly found that Tyr-368 was also phosphorylated. Tyr-368 was not included in our original set of mutants, because this site is not present in Cbl 3. Tyrosine 368 is conserved in all other Cbl family members, however, and we show that phosphorylation at this site also contributes to E3 activation. Tyrosine 368 can also be mutated to glutamate without loss of TKB domain function, and Y368E-Y371E double mutants appear to have increased constitutive E3 activity over the single point mutants.

Our data support the earlier conclusions by Levkowitz et al. that phosphorylation of Tyr-371 plays a key role in activating the E3 activity of c-Cbl. How can we reconcile these data with Zheng et al.’s structural studies? It must be noted that x-ray crystallographic studies of necessity provide a static picture of a stable conformation. The extremely powerful and detailed information provided by crystallography is unable to reveal dynamic movements of a protein in solution. We would argue then, that there must be sufficient flexibility in the conformation of c-Cbl for the internally buried Tyr-371 residue to somehow become available for phosphorylation. One possibility is that, in solution, the structure proposed by Zheng et al. might be in equilibrium with some other conformation of c-Cbl in which Tyr-371 is more accessible. Alternatively, binding interactions between c-Cbl and another protein, perhaps Src, might alter the conformation of c-Cbl sufficiently to provide access for phosphorylation. Zheng et al. state that, if Tyr-371 were to be phosphorylated, this would “result in significant structural change in the linker-TKB and linker-E2 interfaces.” Our studies do not provide the level of resolution necessary to confirm these predictions. Nonetheless, our data do provide direct evidence that tyrosine phosphorylation of c-Cbl alters its conformation. Furthermore, our Y371E mutant also has an altered conformation, possibly mimicking the effect of tyrosine phosphorylation.

The protease digestion studies performed here indicate that tyrosine phosphorylation of c-Cbl, or mutation of tyrosine 371 to glutamate, both increase the stability of a tryptic fragment of ~38 kDa. How might this be achieved? As discussed earlier, this tryptic fragment is likely to consist of residues 54–382 of c-Cbl, containing the entire TKB and linker helix domains. Introduction of fixed negative charge at residue 371 through phosphorylation or mutation is expected to disrupt the interaction of residue 371 with threonine 227, and the negatively charged side chain would be unlikely to remain in the hydrophobic interior occupied by Tyr-371 in the crystal structure. For tyrosine phosphorylation or glutamate mutation at residue 371 to stabilize such a peptide, one mechanism would be the formation of a salt bridge between the negative charge at residue 371 and a nearby positively charged residue. Examination of the published crystal structures does not reveal any nearby positive charges in the hydrophobic interior region occupied by the Tyr-371 side chain. However, rotation of the linker helix could allow the side chain of residue 371 to reach the surface where it might form a salt bridge with arginine 343 or perhaps with another of the several positively charged residues located in this surface vicinity. More detailed structural studies of our Y371E mutant or the Y368E/Y371E double mutant might be of some interest as a model for structural changes occurring upon phosphorylation.

Our data demonstrating conformational changes induced in c-Cbl by tyrosine phosphorylation are in agreement with suggestive earlier findings by Yokouchi et al. (25). These authors performed in vitro binding studies with purified GST-UbcH7, purified c-Cbl, ATP, and SF9 cell extracts containing or lacking Src. In the absence of Src, c-Cbl was observed to co-precipitate with GST-UbcH7. When cell extracts containing Src were employed in this system, c-Cbl no longer co-precipitated with GST-UbcH7. The authors had previously shown that under similar conditions, c-Cbl was tyrosine-phosphorylated by Src. (Furthermore, our laboratory has also published data indicating that Src family kinases phosphorylate c-Cbl (26).) Omission of ATP from the reactions, or pretreatment of the SF9 cells with the Src inhibitor PP1, restored c-Cbl binding to GST-UbcH7 and led to the presence of variable amounts of Src co-precipitating in the complex. These data lend support to the idea that tyrosine phosphorylation of c-Cbl alters its conformation, as evidenced by its ability to stably bind UbcH7.

Tyrosines 368 and 371 of c-Cbl have also received much attention in another context. Single amino acid deletion of either tyrosine confers transforming ability to c-Cbl (27). Four other transforming mutants of c-Cbl are known: point mutation of tyrosine 371 to alanine, Y371A (28), and the deletion mutants v-Cbl (a truncation after residue 355) (2), 70Z-Cbl (an internal deletion of residues 366–382) (2), and p95Cbl (an internal deletion of residues 366–477) (29). All of the currently known transforming mutants of c-Cbl lack the ability to ubiquitinate activated EGFR while retaining the ability to bind to it. It has therefore been proposed that they act as dominant negative mutants. However, several other mutants that bind to activated EGFR and are unable to ubiquitinate it do not transform cells, including Y371F. Thus it has not been clear what specific features are required for transforming ability in c-Cbl; inability to conjugate ubiquitin is insufficient. Thiens et al. (28) have studied this issue in some detail and note that all of the
known transforming mutants in c-Cbl disrupt the helical linker domain between the TKB domain and the RING finger. This linker helix also makes contacts with bound UbcH7. Thi~\textit{en et al.} (28) suggest that disruption of specific interactions between TKB, RING, and bound UbcH7 may be critical for transforming ability rather than simply the loss of E3 activity. We have shown here that phosphorylation of Tyr-368 and Tyr-371, both located in this linker helix region, is critical for altering the conformation of c-Cbl and activating ubiquitin ligase activity. Clearly, further work will be required to fully understand both the normal cellular functions of c-Cbl and how disruptions of c-Cbl function can transform cells.

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