Leucine Zipper-mediated Homodimerization of the Adaptor Protein c-Cbl

A ROLE IN c-Cbl's TYROSINE PHOSPHORYLATION AND ITS ASSOCIATION WITH EPIDERMAL GROWTH FACTOR RECEPTOR

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The 120-kDa proto-oncogenic protein c-Cbl is a multidomain adaptor protein that is phosphorylated in response to the stimulation of a broad range of cell surface receptors and participates in the assembly of signaling complexes that are formed as a result of the activation of various signal transduction pathways. Several structural features of c-Cbl, including the phosphotyrosine-binding domain, proline-rich domain, and motifs containing phosphotyrosine and phosphoserine residues, mediate the association of c-Cbl with other components of these complexes. In addition to those domains that have been demonstrated to play a role in the binding of c-Cbl to other signaling molecules, c-Cbl also contains a RING finger motif and a putative leucine zipper. In this study, we demonstrate that the previously identified putative leucine zipper mediates the formation of Cbl homodimers. Using the yeast two-hybrid system, we show that deletion of the leucine zipper domain is sufficient to abolish Cbl homodimerization, while Cbl mutants carrying extensive N-terminal truncations retain the ability to dimerize with the full-length Cbl. The requirement of the leucine zipper for the homodimerization of Cbl was confirmed by in vitro binding assays, using deletion variants of the C-terminal half of Cbl with and without the leucine zipper domain, and in cells using Myc and green fluorescent protein (GFP) N-terminal-tagged Cbl variants. In cells, the deletion of the leucine zipper caused a decrease in both the tyrosine phosphorylation of Cbl and its association with the epidermal growth factor receptor following stimulation with epidermal growth factor, thus demonstrating a role for the leucine zipper in c-Cbl's signaling functions. Thus, the leucine zipper domain enables Cbl to homodimerize, and homodimerization influences Cbl's signaling function, modulating the activity of Cbl itself and/or affecting Cbl's associations with other signaling proteins in the cell.

Characterization of the mechanisms by which multiprotein signaling complexes are assembled around or downstream of activated cell surface receptors is essential for understanding how signal transduction pathways operate. In addition to receptor and nonreceptor tyrosine kinases and their substrates, adaptor proteins, with their specialized modular domains, participate in the assembly of such multiprotein complexes, recruiting signaling molecules into specific networks (1). Accumulating experimental data indicates that the c-Cbl protein is such an adaptor molecule, with diverse binding domains that contribute to the assembly of signaling complexes involved in various signal transduction pathways (2–4). c-Cbl is a 120-kDa cytosolic protein that was originally identified as a cellular homologue of transforming v-Cbl, an extensively C-terminally truncated form of Cbl that is implicated in the development of pre-B cell lymphomas and myelogenous leukemias in mice (5, 6). It is ubiquitously expressed, with the highest levels found in cells of hematopoietic origin (6).

c-Cbl has been shown to be a major substrate of protein-tyrosine kinases following activation of a broad range of cell surface receptors. Thus, tyrosine-phosphorylation of c-Cbl has been demonstrated in response to EGF (1), platelet-derived growth factor, fibroblast growth factor, nerve growth factor, colony-stimulating factor-1, granulocyte-macrophage colony-stimulating factor, and erythropoietin, as well as upon the stimulation of the Fcγ receptor, T and B cell antigen receptors, c-Kit, integrins, and receptors for interferon α, interleukin-3, insulin, and prolactin (6–15). Furthermore, tyrosine phosphorylation of Cbl has been also observed in v-Src-, v-Abl-, and BCR-Abl-transformed cells (6, 7, 16). These tyrosine phosphorylation events may then lead to c-Cbl’s incorporation into molecular assemblies through the binding of the phosphorylated sequences to the Src homology 2 or phosphotyrosine-binding (PTB) domains of a wide range of other signaling molecules, as has been shown for Crk family members; protein-tyrosine kinases Fyn, Lck, and Abl; the β5 subunit of PI 3-kinase; phospholipase Cγ; and the nucleotide exchange factor Vav (2, 9, 16–21). Conversely, c-Cbl also contains a PTB domain in its N-terminal half that binds to the activated ZAP-70 protein kinase and phosphorylated EGF and platelet-derived growth factor receptors (22–24). In addition to these phosphotyrosine-dependent interactions, stimulation of the T cell antigen receptor has been reported to induce serine phosphorylation of c-Cbl, which in turn results in the binding of c-Cbl to the members of the 14-3-3 family of proteins (25).

However, c-Cbl is a multidomain protein with many potential binding sites that can mediate protein associations through mechanisms other than phosphotyrosine- or phosphoserine-de-
pendent interactions. Domains of c-Cbl that have been demonstrated or predicted to be engaged in protein associations include a RING finger in the middle of the molecule, an extensive proline-rich region in the C-terminal half of the protein, and a putative leucine zipper at the C terminus (6). Indeed, c-Cbl has been shown to associate through the proline-rich region with numerous Src homology 3 domain-containing proteins, including the adaptor molecules Grb2 and Nck and the protein kinases Fyn, Lyn, Lck, Src, and Bruton’s tyrosine kinase (7, 9, 16–18, 20, 26–28). While no involvement of the RING finger of c-Cbl in protein interactions has yet been reported, it is known that small deletions or point mutations in this region activate the transforming potential of c-Cbl (6), and we have found that this domain binds the ubiquitin-conjugating protein UbcH7. Furthermore, the N-terminal half of c-Cbl contains a nuclear localization signal that could play a role in the transforming activity of v-Cbl (6).

Together, these findings point to an important general role of c-Cbl in signaling downstream of cellular receptors coupled to tyrosine kinases, but specific biological functions of this molecule remain unclear. Recent reports indicate, however, that c-Cbl has the ability, through direct interactions, to regulate the function of signaling proteins. Based on genetic studies, SLI-1, the homologue of Cbl in Caenorhabditis elegans, was identified as a negative regulator of the LET-23 receptor tyrosine kinase, the homologue of the mammalian EGF receptor (29). A similar regulatory function has been postulated for D-Cbl, the Drosophila homologue of Cbl (30). In mammalian cells, expression of transforming mutants of Cbl (v-Cbl and 70Z-Cbl) up-regulates the signaling through platelet-derived growth factor receptor α, and the requirement for the PTB domain in this process has been demonstrated (24). Similarly, the expression of 70Z-Cbl has been shown to induce increases in both tyrosine phosphorylation and kinase activity of the EGF receptor (31). Furthermore, overexpression of Cbl inhibits the activity of the Syk nonreceptor tyrosine kinase, with which Cbl forms a complex (32). Finally, the Cbl PTB domain has been found to bind to the in vivo negative regulatory phosphorylation site of ZAP-70 (33). Based on these data, the PTB domain-dependent role of Cbl as a negative regulator of receptor and nonreceptor tyrosine kinases has been postulated (33). Thus, c-Cbl may function both as a negative regulator of tyrosine kinases and as a multifunctional adaptor protein.

All of these earlier studies have focused on the interactions of c-Cbl with other signaling proteins but have not considered the possibility of its self-association. Some of c-Cbl’s protein-binding domains, such as a RING finger and a putative leucine zipper, are, however, known to mediate the formation of homodimers in other proteins. We therefore examined the ability of c-Cbl in protein interactions has yet been reported, it is known that small deletions or point mutations in this region activate the transforming potential of c-Cbl (6), and we have found that this domain binds the ubiquitin-conjugating protein UbcH7. Furthermore, the N-terminal half of c-Cbl contains a nuclear localization signal that could play a role in the transforming activity of v-Cbl (6).

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EGF or vehicle for 3 min at 37 °C, washed with ice-cold phosphate-buffered saline, and lysed in mRIPA.

**Purification of Fusion Proteins—**T7-Tag-Cbl and GST-Cbl fusion proteins were expressed in BL21(DE3) cells (Novagen) transformed with pET-28c-Cbl and pGEX-5X-3-Cbl constructs, respectively. T7-Tag-Cbl fusion proteins were solubilized using His-Bind resin and the His-Bind buffer kit supplied by Novagen following the manufacturer's procedure for the purification under native conditions. Specifically, 500 ml of culture was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h, the binding of the fusion protein to His-Bind resin was performed in a batchwise fashion for 2 h at 4 °C, and 0.1% Triton X-100 was included in the buffer to reduce adsorption binding. GST, GST-Grb2, and GST-Cbl fusion proteins were purified from 500 ml of bacterial culture induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 4.5 h, basically following the procedure described elsewhere (34).

Fusion proteins were bound to glutathione-Sepharose 4B (Amersham Pharmacia Biotech) by incubation batchwise on the rocking plate for 6 h at 4 °C, and the protein was eluted with 20 mM reduced glutathione (Pharmacia Biotech) by incubation batchwise on the rocking plate for 4.5 h, basically following the procedure described elsewhere (34). Fusion proteins were subsequently concentrated on Centricon concentrators (Amicon), and the concentration of proteins was evaluated visually on SDS-polyacrylamide gels stained with Coomassie Brilliant Blue R-250, comparing with bovine serum albumin standards.

**Immunoprecipitations—**250 μg of cell lysate protein was incubated overnight at 4 °C in mRIPA containing 0.5 μg of mouse monoclonal c-Myc antibody (Santa Cruz Biotechnology) or green fluorescent protein (GFP) antibody (CLONTECH) and 25 μl of protein G-Sepharose beads (Calbiochem). Beads were then washed three times with mRIPA, and protein was analyzed by Western blotting.

**Western/Far Western Blotting—**The following antibodies were used: mouse monoclonal antibody (mAb) anti-phosphotyrosine (Upstate Biotech), anti-c-Myc mAb (Santa Cruz Biotechnology), rabbit anti-EGFR polyclonal antibody (Santa Cruz Biotechnology), anti-GFP mAb (CLONTECH), anti-T7-Tag mAb (Novagen), and horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG antibodies (Promega). Total cell lysates, immunoprecipitates, or purified GST, GST-Grb2, and GST-Cbl fusion proteins (amounts indicated in the figure legends) were heated for 10 min at 37 °C in standard SDS-sample buffer and separated on 10% SDS-polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes (BA85; pore size, 0.45 μm) (Schleicher & Schuell), and the filters were incubated for 2 h at room temperature in 5% milk, TBST buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20). For Far Western blots, filters were probed with purified T7-Tag-Cbl fusion proteins at a concentration of 2 μg/ml in 2% bovine serum albumin, TBST, 1 mM dithiothreitol for 1 h at room temperature, washed extensively five times with TBST containing 0.3% Tween 20, and then analyzed by sequential incubation with mAb anti-T7-Tag (1:10,000 dilution) and horseradish peroxidase-conjugated anti-mouse IgG antibody (1:20,000 dilution) in 2% bovine serum albumin, TBST for 30 min each. Immunoblotting and total cell lysates or purified GST, GST-Grb2, and GST-Cbl fusion proteins were visualized by immunoblotting with the appropriate primary antibody (1:1000 dilution) and then a horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody. All blots were developed using enhanced chemiluminescence reagents from Amersham Pharmacia Biotech. To verify the quality of all blotts, proteins were visualized on the filters by staining with 0.2% Ponceau S in 3% trichloroacetic acid.

**In Vitro Binding—**Purified GST and GST-Cbl fusion proteins (1 or 2 μg, as indicated in the figure legends) were bound to glutathione-Sepharose 4B (Amersham Pharmacia Biotech) by agitation on the rocking plate for 2 h at 4 °C in 0.5 ml of HNTG buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 0.1% Triton X-100) containing 1% bovine serum albumin and 1 mM dithiothreitol with or without competing T7-Tag-Cbl-LZ fusion protein. At this time, the test T7-Tag-Cbl fusion protein was added to the tubes, and the incubation continued for 1.5 h at room temperature. Beads were washed three times with 1 ml of HNTG containing 1% Triton X-100 and boiled in SDS-sample buffer, and the samples were electrophoresed on 12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose filters and blotted with mAb anti-T7-Tag as for Far Western blotting.

**RESULTS**

For the two-hybrid assay, full-length c-Cbl and mutant variants of c-Cbl were fused to both the LexA DNA binding domain and the transcription activation domain of the VP16 protein, and pairs of LexA-Cbl and VP16-Cbl hybrid proteins were coexpressed in yeast. The binding of two hybrid proteins to each other results in transactivation of two reporter genes: the yeast HIS3 gene and the bacterial lacZ gene, which are driven by promoters fused to multiple binding sites for LexA. Binding is therefore detected by the growth of yeast in the medium lacking histidine and by induction of the activity of β-galactosidase.

We first tested the ability of full-length human c-Cbl (Cbl-FL) to form homodimers. Coexpression in yeast of the LexA-Cbl-FL and VP16-Cbl-FL hybrid proteins resulted in both growth in the medium lacking histidine and induction of high β-galactosidase activity, indicating strong homodimerization (Fig. 2, column 1). We next examined whether the domain(s) of c-Cbl that are responsible for homodimer formation reside in the N- or C-terminal half of the Cbl molecule. For this purpose, constructs expressing the N-terminal half (Cbl-N half) and the C-terminal half (Cbl-C half) (see Fig. 1) were tested for the homodimer interaction in the two-hybrid assay. As shown in Fig. 2, yeast that coexpressed LexA-Cbl-N half and VP16-Cbl-N half failed to grow on the his- plates or to express β-galactosidase, demonstrating that the N-terminal half of Cbl does not have the capability to form homodimers and that deletion of sequences in the C-terminal half abolished the homodimer interaction. The dimerization of the C-terminal half could not be demonstrated directly, however, since the coexpression of the LexA-Cbl-C half with the VP16 protein alone activated both the HIS3 and lacZ reporter genes, resulting in high backgrounds in both assays. Thus, the Cbl-C half when fused to the LexA DNA binding domain showed the properties of an activator of transcription, preventing its use in a yeast two-hybrid assay.

In order to identify the sequences in the C-terminal half that mediate the homodimerization, we generated a series of mutants of c-Cbl (as shown schematically in Fig. 1) in which specific domains localized within the C-terminal half were deleted from the full-length Cbl construct: Cbl-Δ (Ac + LZ), in which both acidic and leucine zipper domains were deleted; Cbl-ΔAc, which lacked the acidic domain; and Cbl-ΔLZ, which lacked the leucine zipper domain. Each construct was fused to both LexA and VP16 for analysis of homodimerization in the two-hybrid assay. As shown in Fig. 2, Cbl-Δ (Ac + LZ), which consists of the N-terminal half and the proline-rich domain (see Fig. 1), did not homodimerize, indicating that either the acidic domain or the proline-rich domain are not sufficient for homodimerization.
domain or the putative leucine zipper domain carries the sequences responsible for homodimer formation. Cbl-DΔAc, from which residues 711–834 were deleted, showed an ability to form homodimers that was only slightly weaker than the homodimerization of Cbl-FL, as evaluated by the levels of β-galactosidase activity and growth of yeast on the his− plates (Fig. 2). In contrast, deletion of only 50 amino acids at the C terminus (Cbl-DΔLZ) was sufficient to abolish the homodimerization of Cbl completely (Fig. 2). As noted above, this region of Cbl has been previously defined as a putative leucine zipper domain, based on the presence of six heptad repeats of hydrophobic amino acids (6). To exclude the possibility that activation of reporter genes by some of the Cbl constructs was a result of nonspecific protein interactions, two kinds of negative control experiments were performed: 1) LexA-Cbl fusion proteins were tested for the interaction with VP16 protein itself, and 2) VP16-Cbl hybrids were coexpressed with LexA-lamin fusion protein. None of the Cbl constructs presented in Fig. 2, including Cbl-FL, induced growth in his− medium or β-galactosidase activity in either of the controls. However, fragments of Cbl-C half that contained the acidic domain, such as Cbl-C half, ΔLZ, Cbl-Ac + LZ, and Cbl-Ac (see Fig. 1), like the complete Cbl-C half, behaved as transcription activators when fused to LexA and therefore could not be tested for homodimer interaction in the two-hybrid assay.

The results of the two-hybrid assay demonstrated that the sequences present within the putative leucine zipper domain at the C terminus of Cbl are responsible for the ability of the protein to form homodimers. In order to examine the role of the leucine zipper in homodimerization in more detail, we then analyzed the interaction of full-length Cbl, expressed as LexA-Cbl-FL fusion, with various deletion mutants and short domains of Cbl, expressed as fusions with VP16. As shown in Fig. 3, all of the C-terminally truncated Cbl mutants, Cbl-DΔLZ, Cbl-DΔAc + LZ, and Cbl-N half, with the shortest truncation being in Cbl-DΔLZ, failed to associate with Cbl-FL, confirming that the leucine zipper domain is absolutely required in order for the homodimer interaction to occur. In contrast, the two N-terminally truncated Cbl mutants, even the small Cbl-Ac + LZ, which consists of only the acidic and leucine zipper domains, had the ability to dimerize with Cbl-FL (see Fig. 3), and these interactions were only slightly weaker than that of the Cbl-FL homodimer. Furthermore, deletion of the leucine zipper domain from Cbl-C half and Cbl-Ac + LZ (Cbl-C half, ΔLZ and Cbl-Ac, respectively) was sufficient to abolish the interaction with Cbl-FL. Finally, as expected, Cbl-DΔAc interacted strongly with Cbl-FL, with the activity of β-galactosidase being even higher than that induced by Cbl-FL homodimer, indicating that deletion of the acidic domain does not suppress the ability of the protein to dimerize (Fig. 3). The results of the β-galactosidase assays presented in Fig. 3 were confirmed by the analysis of yeast growth in the his− medium. None of the VP16-Cbl variants tested for the interaction with LexA-Cbl-FL were able to induce expression of the lacZ and the HIS3 gene when coexpressed with LexA-lamin.

Since the data from the two-hybrid assay indicated that the Cbl protein has the ability to form homodimers when expressed in vivo in yeast cells and that this interaction is leucine zipper-mediated, we then investigated the homodimerization of Cbl by using in vitro binding experiments with fusion proteins. For this purpose, the C-terminal half of Cbl and various truncation mutants of the C-terminal half, with and without the leucine zipper domain, were expressed as fusion proteins with GST or T7-Tag, purified, and then analyzed for direct interaction by Far Western blotting and in vitro binding assays with glutathione-Sepharose beads. For Far Western blotting, GST-tagged fusion proteins containing the C-terminal half of Cbl with or without the leucine zipper domain were first separated on SDS-polyacrylamide gels and transferred to nitrocellulose filters, and then the filters were probed with various T7-Tag containing Cbl mutants (Fig. 4). GST-Grb2 and GST were included on the filters as control proteins. The T7-tagged C-terminal half of Cbl (T7-Tag-Cbl-C half) bound to GST-Cbl-C half but not to GST-Cbl-C half, ΔLZ (Fig. 4A, compare lanes 3 and 4 with lanes 5 and 6). When the blot was probed with the C-terminal half, which lacked the leucine zipper domain (T7-Tag-Cbl-C half, ΔLZ), no binding to either GST-Cbl-C half or GST-Cbl-C half, ΔLZ was observed, as shown in Fig. 4B (lanes 3–6). Thus, removing the leucine zipper from either one of interacting partners abolished the homodimerization (Fig. 4, A, lanes 5 and 6, and B, lanes 3 and 4), indicating that direct interaction of the C-terminal half of Cbl takes place only when the leucine zipper domain is present in both binding partners (Fig. 4A, lanes 3 and 4). Both T7-Tag-Cbl-C half and T7-Tag-Cbl-C half, ΔLZ fusion proteins bound to GST-Grb2 (Fig. 4, A
Dimerization of Cbl Fusion Proteins Can Be Competitively Inhibited by a Leucine Zipper Fusion Protein—To provide further evidence of an *in vitro* association, we investigated the interaction in solution of GST-Cbl proteins with T7-Tag-Cbl fusion proteins. GST-Cbl fusion proteins were bound to the glutathione-Sepharose beads, incubated with the individual T7-Tag-Cbl proteins, and then, after extensive washing, the bound T7-Tag-Cbl proteins were quantified as described under “Experimental Procedures.” As in the filter binding assay, GST-Cbl-C half was found to associate with T7-Tag-Cbl-C half and the shorter variant T7-Tag-Cbl-Ac + LZ (Fig. 5, *top* and *bottom panels, lane 2*), both of which contain the leucine zipper domain, but not with the counterparts lacking the leucine zipper, T7-Tag-Cbl-C half, ∆LZ and T7-Tag-Cbl-Ac (Fig. 5, *top* and *bottom panels, lane 6*). Similarly, the association was abolished when the immobilized GST-Cbl fusion protein lacked the leucine zipper (Fig. 5, *lane 7 of both panels*). The binding of the T7-tagged Cbl proteins containing the leucine zipper domain to the immobilized GST-Cbl-C half could be competitively displaced by increasing amounts of T7-Tag-Cbl-LZ protein (Fig. 5, *top* and *bottom panels, compare lane 2 with lanes 3–5*). Thus, Cbl fusion proteins can associate *in vitro* in a leucine zipper-dependent manner.

**c-Cbl Dimerization in HEK293 Cells Is Dependent upon the Leucine Zipper**—In order to determine whether Cbl homodimerization occurs *in vivo*, cells that stably expressed N-terminal EGFP-tagged Cbl-FL were transiently transfected with N-terminal Myc-tagged Cbl-FL and Cbl-ALZ. EGFP-tagged Cbl was then immunoprecipitated from total cell lysates, and the immune complexes were Western blotted for the presence of Myc-Cbl-FL or Myc-Cbl-ALZ. As can be seen in Fig. 6, a protein of approximately 120 kDa, which corresponds to the correct size for Myc-Cbl-FL, was detected in anti-GFP im-

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**Fig. 4.** Leucine zipper-dependent direct binding of GST-Cbl fusion proteins to T7-Tag-Cbl fusion proteins. Purified GST fusion proteins were analyzed for binding with purified T7-Tag-Cbl fusion proteins by Far Western blotting, as described under “Experimental Procedures.” GST (2 µg, A–E, lane 1) and GST-Grb2 (0.15 µg, A–D, lane 2) were included on the gels as controls. A and B, 0.25 and 0.5 µg of GST-Cbl-C half (lanes 3 and 4) and GST-Cbl-C half, ∆LZ (lanes 5 and 6) were probed with T7-Tag-Cbl-C half (A) or T7-Tag-Cbl-C half, ∆LZ (B). C and D, 0.125 and 0.25 µg of GST-Cbl-C half (lanes 3 and 4) and GST-Cbl-C half, ∆LZ (lanes 5 and 6) were probed with T7-Tag-Cbl-Ac + LZ (C) or T7-Tag-Cbl-Ac (D). E, 0.25, 0.5, and 1 µg of GST-Cbl-C half (lanes 2–4) and GST-Cbl-C half, ∆LZ (lanes 5–7) were probed with T7-Tag-Cbl-LZ.
Fig. 5. Dimerization of c-Cbl fusion proteins is competitively disrupted by the leucine zipper domain of Cbl (Cbl-LZ). GST-Cbl-C half (2 μg, top panel; 1 μg, bottom panel) was incubated with T7-Tag-Cbl-C half (2 μg, top panel) or T7-Tag-Cbl-Ac + LZ (1 μg, bottom panel) alone (lane 2) or in the presence of increasing concentrations of T7-Tag-Cbl-LZ (20, 50, and 125 μg, lanes 3–5), and the binding was analyzed as described under “Experimental Procedures.” For negative controls, GST-Cbl-C half was replaced with an equal amount of GST (lane 1) or GST-Cbl-C half, ΔLZ (lane 7), or T7-Tag-Cbl-C half (top panel) and T7-Tag-Cbl-Ac + LZ (bottom panel) were replaced with an equal amount of T7-Tag-Cbl-C half, ΔLZ and T7-Tag-Cbl-Ac, respectively (lane 6).

Fig. 6. Dimerization of c-Cbl in HEK293 cells is dependent upon the presence of the leucine zipper. HEK 293 cells stably expressing EGFP-Cbl-FL were transiently transfected with pBK-Myc (control), pBK-Myc-Cbl-ΔLZ, or pBK-Myc-Cbl-FL, as indicated. EGFP-Cbl-FL was immunoprecipitated from 250 μg of cell lysate protein, and dimerization with Myc-Cbl-FL or Myc-Cbl-ΔLZ was analyzed by immunoblotting with anti-Myc antibody (top panel). To evaluate the immunoprecipitation (IP) of the EGFP-Cbl-FL fusion protein, blots were reprobed with anti-GFP antibody (middle panel). The expression of Myc-Cbl-FL and Myc-Cbl-ΔLZ was assessed by blotting 20 μg of cell lysate protein with anti-Myc antibody (bottom panel). TCL, total cell lysate.

munoprecipitates from cells expressing Myc-Cbl-FL, while no Myc-tagged Cbl-ΔLZ could be detected in immunoprecipitates from cells expressing that protein. Similar amounts of EGFP-Cbl-FL were immunoprecipitated from all lysates, and similar amounts of Myc-Cbl-FL and Myc-Cbl-ΔLZ were detected in the appropriate total cell lysates. These data demonstrate that c-Cbl homodimerizes in mammalian cells in a leucine zipper-dependent manner.

The Leucine Zipper Is Required for Efficient Tyrosine Phosphorylation of c-Cbl and Its Association with the EGFR following Stimulation with EGF—The stimulation of growth factor receptors, such as the EGFR, induces the rapid tyrosine phosphorylation of c-Cbl and the recruitment of c-Cbl to the activated receptors (6). To test whether these events are influenced by the dimerization of c-Cbl, HEK 293 cells were transiently transfected with vector alone, Myc-Cbl-FL, or Myc-Cbl-ΔLZ and, after 36-h serum starvation, stimulated with 100 ng/ml EGF or vehicle for 3 min. Western blot analysis of total cell lysates showed equivalent levels of expression of Myc-tagged Cbl-FL and Cbl-ΔLZ in the appropriate samples (Fig. 7A, bottom panel). In all three lysates, stimulation of the EGFR resulted in a dramatic and comparable increase in tyrosine phosphorylation of proteins over a wide range of molecular weights, including the 110–200-kDa region (Fig. 7A, top panel). A strong 120-kDa band was apparent in the lysates from stimulated cells that had been transfected with Cbl-FL but was not present in control cell lysates or in Cbl-ΔLZ-expressing cells, suggesting that Cbl-FL, but not Cbl-ΔLZ, was being efficiently tyrosine-phosphorylated. To further analyze the phosphorylation and receptor association of Cbl, Myc-tagged Cbl proteins were immunoprecipitated, and the immune complexes were blotted with antibodies to phosphotyrosine, Myc, and the EGFR (Fig. 7B). This analysis confirmed that EGF-induced tyrosine phosphorylation of Cbl-ΔLZ was markedly decreased, relative to the phosphorylation of Cbl-FL. In addition, the amount of
activated EGFR (23) was greatly reduced in the Cbl-LZ sample (Fig. 7B, top panel), despite the fact that the induction of EGFR phosphorylation by EGF was similar in all lysates (Fig. 7A, top panel). Blotting the anti-Myc immunoprecipitates with anti-EGFR (Fig. 7B, bottom panel) confirmed that markedly less of the 170-kDa EGFR was associated with Cbl-LZ than with Cbl-FL. Thus, the leucine zipper-mediated dimerization of c-Cbl appears to be required for the maximally efficient tyrosine phosphorylation of c-Cbl and for the effective binding of c-Cbl to the activated receptor.

**DISCUSSION**

c-Cbl has been previously shown to interact with a wide range of signaling proteins through the engagement of its PTB domain, proline-rich domain and phosphotyrosine- and phosphoserine-containing motifs (2, 7, 9, 18, 20, 22, 23, 25). With the exception of the PTB domain, the other known functional binding sites are localized within the C-terminal half of the molecule. In this study, we demonstrated that another domain in the C-terminal half of c-Cbl, previously identified as a putative leucine zipper domain, mediates the formation of c-Cbl homodimers both in vitro and in vivo. Furthermore, we found that the leucine zipper is the only domain of the Cbl molecule that is absolutely required in order for the homodimer interaction to occur. (A RING finger motif has been identified in the N-terminal half of the molecule, and while it may participate in the associations of c-Cbl with other proteins, it does not mediate c-Cbl homodimerization.) Several lines of evidence support these conclusions. First, in the two-hybrid assay and in HEK293 cells, deletion of the C-terminal leucine zipper domain was sufficient to abolish homodimerization (Fig. 2), and a Cbl variant lacking the leucine zipper was unable to interact with the full-length Cbl (Figs. 3 and 6). Second, a Cbl mutant carrying extensive truncations of N-terminal sequences and composed solely of the acidic and leucine zipper domains retained the ability to dimerize with the full-length Cbl in the two-hybrid system (Fig. 3) and with the C-terminal half of Cbl in the in vitro binding assays (Figs. 4 and 5). Both interactions could be abolished by deleting the leucine zipper domain. Third, the in vitro dimerization of the C-terminal half of Cbl with itself and with the variant composed of the acidic and leucine zipper domains could be competitively disrupted by a recombinant protein containing little more than the leucine zipper domain (Fig. 5). However, although the leucine zipper domain is indispensable for Cbl homodimerization, and sequences upstream of the leucine zipper domain do not themselves show the ability to dimerize, quantitative analysis of the two-hybrid assay suggests that these upstream sequences may contribute to the strength of the homodimerization of the full-length Cbl molecules. Thus, the full-length Cbl homodimer induced higher β-galactosidase activity than did the homodimer of Cbl that lacked the acidic domain (Fig. 2), and the homodimerization of the full-length Cbl was stronger than the interaction of full-length Cbl with either the C-terminal half of Cbl or the fragment composed of the acidic and leucine zipper domains (Fig. 3). Moreover, in the in vitro binding studies with fusion proteins, the interaction of the C-half of Cbl with itself or with the fusion protein containing the acidic and leucine zipper domains was much stronger than the association of Cbl C-half with a short fragment comprised solely of the leucine zipper domain (Fig. 4).

As noted under “Results,” fusion proteins of the LexA DNA binding domain with the C-terminal half of Cbl and deletion variants thereof behaved as activators of transcription in the two-hybrid assay in the presence of the VP16 activation domain alone and for this reason could not be tested for the formation of dimers with the interacting partners. Detailed deletion analysis indicated that the sequences contained within the acidic domain of Cbl are in large part responsible for this effect (data not shown). Similar transactivation by acidic sequences in other proteins has been reported (38). The absence of such an effect with the full-length Cbl-LexA fusion suggests that the acidic domain may be hidden inside the folded protein structure of the full-length molecule and that it reveals its transactivation potential only after removal of the sequences comprising the N-terminal half of Cbl. Furthermore, the dimer of full-length Cbl with the Cbl that lacked the acidic domain (Cbl DAc) induced higher β-galactosidase activity than the full-length Cbl homodimer (Fig. 3). This increased transcription activation might be the result of the exposure of the acidic domain of the full-length Cbl molecule in the dimer formed between Cbl-FL and Cbl DAc.

The leucine zipper domain is an α-helical structure formed by several heptad repeats of hydrophobic residues, usually leucine and isoleucine, that is commonly found in nuclear transcription factors, and its role in promoting the homo- and heterodimerization of these proteins has been well documented (39, 40). Leucine zipper domains have also been identified in many other proteins, mostly protein kinases and cytoskeletal proteins, but their function in these proteins has been less extensively examined. Leucine zipper-mediated homodimerization of some chimeric receptor tyrosine kinases, which are formed as a result of chromosomal rearrangements, is considered to be responsible for their oncogenic activity (41, 42). Moreover, the leucine zipper-dependent homodimerization of both cGMP-dependent protein kinase and ZIP kinase, a serine/threonine kinase, is necessary for their activity (43, 44). Some other serine/threonine protein kinases such as fatty acid-activated protein kinase N and members of the mixed lineage kinase family also contain leucine zipper motifs, but the function of the domain in these proteins has not been elucidated (45–47). In addition to kinases, many cytoskeletal proteins, including myosin, keratin, α-spectrin, microtubule-associated protein, vimentin, and kinectin, have been found to contain leucine zipper motifs (48–51). In the few cases where the function of a leucine zipper in these proteins has been characterized, it also mediates homo- or heterodimerization. Thus, the cytoskeletal, sperm-specific outer dense fiber proteins, OdS27 and OdS84, have been shown to heterodimerize through the engagement of their leucine zipper domains (52), and dystrophin is capable of interacting with tropomin T via its leucine zipper domain (53). Finally, a recent report on AFAP-110 (actin filament-associated protein, 110 kDa) demonstrates that its leucine zipper structure might play an important role in the protein’s self-association, its cellular localization, and its ability to interact with actin filaments (48).

In the case of c-Cbl, our data show that the deletion of the leucine zipper and the resulting loss of Cbl dimerization lead to decreased EGF-induced tyrosine phosphorylation of c-Cbl and c-Cbl-EGFR association, indicating that the leucine zipper-mediated dimerization of c-Cbl has functional significance. Although two tyrosine residues (at positions 871 and 869) are involved in the activation potential only after removal of the sequences comprising the N-terminal half of Cbl. Furthermore, the dimer of full-length Cbl with the Cbl that lacked the acidic domain (Cbl DAc) induced higher β-galactosidase activity than the full-length Cbl homodimer (Fig. 3). This increased transcription activation might be the result of the exposure of the acidic domain of the full-length Cbl molecule in the dimer formed between Cbl-FL and Cbl DAc.
Homodimerization of c-Cbl

association (55) involves phosphotyrosine residues on c-Cbl. Rather, both the diminished tyrosine phosphorylation of Cbl-ΔLZ and the reduced association of Cbl-ΔLZ with the EGFR are likely to be the consequences of the inability of Cbl-ΔLZ to dimerize. The simplest hypothesis is that the lack of dimerization would probably result in the incorporation of only half as many c-Cbl molecules into the EGFR-associated signaling complex, which could in turn result in half the amount of c-Cbl being phosphorylated by the activated EGFR. However, both the level of Cbl-ΔLZ phosphorylation and the amount of EGFR that is associated with Cbl-ΔLZ appear to be less than half of the amounts observed with Cbl-FL. It may therefore be that the direct association of c-Cbl with the EGFR is stronger for the Cbl dimer than for the monomer or that the presence of two sets of binding domains on the c-Cbl dimer allows the formation of more indirect links (for example, by Grb2), which stabilize the interaction.

c-Cbl is, to our knowledge, the first adaptor protein that is demonstrated to have the ability to homodimerize through a leucine zipper-dependent mechanism. Nevertheless, the homodimerization of adaptor and scaffold proteins through other types of binding domains has been previously reported. Dimerization of Ste 5, a scaffold protein for the components of the mitogen-activated protein kinase cascade in *Saccharomyces cerevisiae*, is mediated by the RING-H2 domain (in contrast to the lack of involvement of the c-Cbl RING finger in its homodimerization) and is essential for the regulation of the pheromone mating response (56, 57). Members of the ubiquitous 14-3-3 family of eukaryotic adaptor proteins, which interact with numerous signaling molecules with distinct functions, exist and act both as homodimers and as heterodimers with other isoforms within the family (58, 59). The recently identified multidomain adaptor protein HEF-1, which is involved in cell adhesion-related signaling pathways (60), homodimerizes via a helix-loop-helix domain that is also required for the biological activity of HEF-1 protein. Finally, members of the Bcl-2 family of regulators of apoptosis, which function both as ion channels and as adaptor/docking proteins, are known to homodimerize and heterodimerize with other family members, a property that is critical for the biological functions of these proteins (61).

Thus, homo- and heterodimerization of adaptor proteins occur within a variety of signal transduction pathways and participate in the regulation of several molecular assemblies. In the case of an adaptor protein such as c-Cbl, the ability to homodimerize might have several consequences for its activity and biological function. Dimeric adaptors could, as suggested previously (1), function as molecular bridges to juxtapose proteins that do not associate directly and/or that bind to the same domain(s) within the adaptor. Dimer formation could also uncover or mask specific functional domains of c-Cbl as a result of conformational changes that might be induced by dimerization. For example, accessibility of the major tyrosine phosphorylation sites, located within the acidic domain in close proximity to the leucine zipper (54), could be altered, thereby changing the kinetics of phosphorylation or dephosphorylation and/or affecting Cbl’s association with other signaling proteins (another possible explanation of our data). In addition, if dimerization is regulated, for example by tyrosine phosphorylation, in the relative amounts of the monomer and dimer could thus serve as a mechanism to modulate c-Cbl activities and functions, such as c-Cbl’s known negative regulatory effect on some protein kinases. It will thus be important to understand how formation of the homodimer is controlled.

Finally, the presence of a functional leucine zipper opens the possibility that Cbl heterodimerizes with other leucine zipper-containing proteins, such as the numerous serine/threonine kinases and cytoskeletal proteins discussed earlier, thereby increasing enormously the number of potential Cbl binding partners. Such interactions could be crucial to Cbl’s normal function. For example, it is possible that the absence of the leucine zipper allows v-Cbl to translocate to the nucleus rather than maintaining the normal cytoplasmic location of c-Cbl. Identifying the molecules that specifically interact with Cbl through the leucine zipper-mediated mechanism will be an important goal of future studies.

In conclusion, we have shown that the putative leucine zipper domain at the C terminus of c-Cbl mediates the formation of homodimers and that the homodimerization of c-Cbl is required for the efficient tyrosine phosphorylation of this protein and its association with the EGFR. Homodimerization may be important for c-Cbl’s association with other signaling proteins in the cell as well. Further investigation of the role of the leucine zipper and c-Cbl homodimerization are thus likely to provide additional important insights into the biology of this signaling protein.

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REFERENCES

1. Pawson, T., and Scott, J. D. (1997) *Science* 278, 2075–2080
2. Reedquist, K. A., Fukazawa, T., Panchamoorthy, G., Langdon, W. Y., Shoelson, S. E., Druker, B. J., and Band, H. (1996) *J. Biol. Chem.* 271, 8435–8442
3. Smit, L., Van der Horst, G., and Borst, J. (1996) *J. Biol. Chem.* 271, 8564–8569
4. Gerhart, F., Garbay, C., and Bertoglio, J. (1998) *J. Biol. Chem.* 273, 3986–3992
5. Blake, T. J., Shapiro, M., Morse, H. C., III, and Langdon, W. Y. (1991) *Oncogene* 6, 653–657
6. Langdon, W. Y. (1995) *Aust. N. Z. J. Med.* 25, 859–864
7. Tanaka, S., Neif, L., Baron, R., and Levy, J. B. (1995) *J. Biol. Chem.* 270, 14347–14351
8. Chin, H., Saito, T., Arai, A., Yamamoto, K., Kiamiya, R., Miyasaka, N., and Miura, O. (1997) *Biochem. Biophys. Res. Commun.* 239, 412–417
9. Fukazawa, T., Reedquist, K. A., Trub, T., Soltsof, S., Panchamoorthy, G., Druker, B., Cantley, L., Shoelson, S. E., and Band, H. (1995) *J. Biol. Chem.* 270, 19141–19150
10. Panchamoorthy, G., Fukazawa, T., Miyake, S., Soltsof, S., Reedquist, K., Druker, B., Shoelson, S., Cantley, L., and Band, H. (1996) *J. Biol. Chem.* 271, 3187–3194
11. Uddin, S., Gardiziola, C., Dangat, A. Y., and Platnaias, L. C. (1996) *Biochem. Biophys. Res. Commun.* 235, 833–838
12. Ojaniemi, M., Martin, S. S., Delé, F., Olefsky, J. M., and Vuori, K. (1997) *J. Biol. Chem.* 272, 3788–3794
13. Anderson, S. M., Burton, E. A., and Koch, B. L. (1997) *J. Biol. Chem.* 272, 739–745
14. Ribon, V., and Saltiel, A. R. (1997) *Biochem. J.* 324, 839–845
15. Hunter, S., Koch, B. L., and Anderson, S. M. (1997) *Mol. Endocrinol.* 11, 1213–1222
16. Jain, S. K., Langdon, W. Y., and Vartivorkis, L. (1997) *Oncogene* 14, 2217–2228
17. Buday, L., Klwaja, A., Sipeki, S., Faraghi, A., and Downward, J. (1996) *J. Biol. Chem.* 271, 6159–6163
18. Donzovan, J. A., Wang, R. L., Langdon, W. Y., and Samelson, L. E. (1994) *J. Biol. Chem.* 269, 22921–22924
19. Sattler, M., Salgia, R., Okuda, K., Uemura, N., Durstin, M. A., Pines, E., Xu, G., Li, L., Prasad, K. V., and Griffin, J. D. (1996) *Oncogene* 12, 839–846
20. Meisner, H., Conway, B. R., Hartley, D., and Czeizel, M. P. (1995) *Cell. Biol. Med.* 15, 3571–3578
21. Marengere, L. E. M., Mirotsos, C., Koskierska, L., Veillette, A., Mak, T. W., and Pennninger, J. M. (1997) *J. Immunol.* 159, 70–76
22. Luster, M. L., Jr., Reedquist, K. A., Miyake, S., Langdon, W. Y., and Band, H. (1996) *J. Biol. Chem.* 271, 24063–24068
23. Thien, C. B., and Langdon, W. Y. (1997) *Oncogene* 14, 2239–2249
24. Bonita, D. P., Miyake, S., Luster, M. L., Jr., Langdon, W. Y., and Band, H. (1997) *Mol. Cell. Biol.* 17, 4597–4610
25. Liu, Y. C., Liu, Y. H., Elly, C., Yoda, K., Lipkowitz, S., and Altman, A. (1997) *J. Biol. Chem.* 272, 9979–9985
26. Rivero-Lezcano, O. M., Sameshima, J. H., Marcilla, A., and Robbins, K. C. (1994) *J. Biol. Chem.* 269, 17363–17366
27. Marcilla, A., Rivero-Lezcano, O. M., Agarwal, A., and Robbins, K. C. (1995) *J. Biol. Chem.* 270, 9115–9120
28. Cory, G. O. C., Lovering, R. C., Hinshelwood, S., MacCarthy-Morrogh, L., Levinsky, R. J., and Kinnon, C. (1995) *Exp. Med.* 182, 611–615

3 S. Law and E. Golemis, personal communication.
