The Evolutionarily Conserved N-terminal Region of Cbl Is Sufficient to Enhance Down-regulation of the Epidermal Growth Factor Receptor

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The mammalian proto-oncoprotein Cbl and its homologues in Caenorhabditis elegans and Drosophila are evolutionarily conserved negative regulators of the epidermal growth factor receptor (EGF-R). Overexpression of wild-type Cbl enhances down-regulation of activated EGF-R from the cell surface. We report that the Cbl tyrosine kinase-binding (TKB) domain is essential for this activity. Whereas wild-type Cbl enhanced ligand-dependent EGF-R ubiquitination, down-regulation from the cell surface, accumulation in intracellular vesicles, and degradation, a Cbl TKB domain-inactivated mutant (G306E) did not. Furthermore, the transforming truncation mutant Cbl-N (residues 1–357), comprising only the Cbl TKB domain, functioned as a dominant negative protein. It colocalized with EGF-R in intracellular vesicular structures, yet it suppressed down-regulation of EGF-R from the surface of cells expressing endogenous wild-type Cbl. Therefore, Cbl-mediated down-regulation of EGF-R requires the integrity of both the N-terminal TKB domain and additional C-terminal sequences. A Cbl truncation mutant comprising amino acids 1–440 functioned like wild-type Cbl in down-regulation assays. This mutant includes the evolutionarily conserved TKB and RING finger domains but lacks the less conserved C-terminal sequences. We conclude that the evolutionarily conserved N terminus of Cbl is sufficient to effect enhancement of EGF-R ubiquitination and down-regulation from the cell surface.

Tyrosine phosphorylation provides a basic mechanism of signal transduction in the cells of higher eukaryotes. Ligand binding to cell surface receptors induces tyrosine phosphorylation by either of two mechanisms as follows: activation of the cytoplasmic kinase domains of receptor tyrosine kinases, or activation of non-receptor tyrosine kinases associated with cell surface receptors. Because the triggering of a particular tyrosine phosphorylation cascade by varied stimuli may lead to cell differentiation, proliferation, or death, and because deregulated tyrosine kinases can be oncogenic, tight regulation of tyrosine kinases is critical for the maintenance of cellular homeostasis. Consequently, identification of cellular proteins that modulate the intensity or duration of tyrosine kinase signaling and defining their mechanisms of action are areas of significant interest in cell biology. Recent studies have implicated the proto-oncoprotein Cbl as a regulator of protein tyrosine kinase function (reviewed in Refs. 1–6).

The c-cbl proto-oncogene was first identified as the cellular homologue of the murine retroviral oncogene v-cbl (7). Its gene product, Cbl, is a 906-amino acid protein comprising two distinct regions, Cbl-N and Cbl-C. Cbl-N consists of amino acids 1–357; this region corresponds to the Cbl sequences retained in the v-cbl oncogene and is sufficient to transform NIH 3T3 cells (8). Cbl-N is evolutionarily conserved (9–11) and has been shown to bind a number of autophosphorylated tyrosine kinases in a phosphotyrosine-dependent manner (12, 13). Cbl-N was previously referred to as a phosphotyrosine-binding domain. However, it has been shown to consist of a four-helical domain, an EF hand, and an incomplete SH2 domain, which together constitute a phosphotyrosine-binding platform (14), and is now designated a tyrosine kinase-binding (TKB) domain (6).

The Cbl sequences C-terminal to the TKB domain (Cbl-C) comprise several structural units that suggest function. These include the following: an evolutionarily conserved RING finger (15); a nonconserved proline-rich region capable of interacting with the Src homology 3 (SH3) domains of Src family kinases, the Grb2 and Nck adapter proteins, and a protein of unknown function, CAP; and a leucine zipper, found in mammalian Cbl but not in the Caenorhabditis elegans or Drosophila homologues. Cbl is a ubiquitous and prominent substrate for tyrosine kinases and contains a number of tyrosine residues within the Cbl-C region that, when phosphorylated, serve as docking sites for the SH2 domains of other signaling proteins (16–21). Whereas these structural features indicate a role for Cbl as an

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The abbreviations used are: TKB, tyrosine kinase-binding; EGF, epidermal growth factor; EGF-R, EGF receptor; HA, hemagglutinin; mAb, monoclonal antibody; HEK, Human embryonic kidney; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PFDV, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; PDGF-R, platelet-derived growth factor receptor; MFI, mean fluorescence intensity; GFP, green fluorescent protein.

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The basic biological process of ligand-induced receptor tyrosine kinase down-regulation is a prominent feature of signaling complexes. Recent investigations have established that Cbl functions as a negative regulator of receptor and non-receptor tyrosine kinases (13, 22-28). A prominent and evolutionarily conserved target of the negative regulatory effect of Cbl is the epidermal growth factor receptor (EGF-R or ErbB-1), a well-studied prototype of receptor tyrosine kinases that mediate cell proliferation and differentiation in response to growth factors.

The cytoplasmic tyrosine kinase domain of the EGF-R undergoes activation and trans-phosphorylation upon the binding of ligand to the EGF-R extracellular domain. The induced phosphorylation creates docking sites for a variety of proteins. One such protein is Cbl, which undergoes ligand-dependent tyrosine phosphorylation and association with the EGF-R (10, 11, 24, 29-35). The EGF-induced association of Cbl with EGF-R is apparently mediated directly via Cbl TKB domain binding to autophosphorylated sites on the receptor, as well as indirectly via the Grb2 adapter protein (31, 33).

For Cbl to function as a biologically relevant regulator of EGF-R, it must be capable of diminishing ligand-dependent EGF-R activity. The evidence for Cbl as a biologically relevant regulator of EGF-R derives from in vivo and as well as in vitro studies. SLI-1, the C. elegans Cbl homologue, was identified by virtue of a loss-of-function mutation in its gene (sli-1) that rescues vulva development in worms expressing a hypomorphic allele of let-23, which encodes the homologue of mammalian EGF-R (36). One reported loss-of-function mutation in SLI-1 is a single amino acid substitution (G315E) in the conserved TKB domain (36). Expression of D-Cbl (the Drosophila Cbl homologue) from a heat shock protein promoter results in reduced R7 photoreceptor development, a process mediated through EGF-R signaling (10). Finally, the mammary glands of Cbl−/− mice are abnormal, with increased ductal density and branching suggesting (10). Finally, the mammary glands of Cbl−/− mice are abnormal, with increased ductal density and branching suggestive of aberrant signaling through EGF-R and/or related receptors (37).

In studies in vitro cell culture model systems extend these observations and provide evidence for direct functional effects of Cbl on the EGF-R. Whereas overexpression of exogenous Cbl decreases the autophosphorylation of EGF-R in NIH 3T3 cells, Cbl antisense transfection increases both EGF-R autophosphorylation and subsequent JAK-STAT activity (25). Interestingly, overexpression of an oncogenic form of Cbl, 70Z/3, in NIH 3T3 cells enhances the activity of EGF-R, possibly by functioning as a dominant negative protein (24, 29). Together, these studies suggest that Cbl functions as a negative regulator of EGF-R signaling. However, potential mechanisms of Cbl-dependent negative regulation of the EGF-R and other receptor tyrosine kinases had not been defined until recently.

Our laboratory demonstrated that overexpression of wild-type Cbl in NIH 3T3 cells led to enhanced ligand-induced ubiquitination and degradation of the platelet-derived growth factor receptor (PDGFR-α) (27), suggesting that Cbl’s negative regulation of other receptor tyrosine kinases, including EGF-R, might be mediated via receptor degradation. This possibility was confirmed recently by Yarden and colleagues (28), who showed that overexpression of wild-type Cbl in Chinese hamster ovary cells led to increased ligand-dependent EGF-R internalization, ubiquitination, and degradation.

We report here that Cbl-mediated down-regulation of EGF-R requires a functional TKB domain. When expressed by itself, the TKB domain (Cbl-N) acts as a dominant negative mutant, indicating a critical role for additional C-terminal Cbl sequences. Enhanced down-regulation of activated EGF-R is effected by Cbl amino acids 1–440. This truncated protein contains the TKB and RING finger domains but lacks multiple domains from the Cbl C terminus. Thus, the N-terminal evolutionarily conserved sequences of Cbl are sufficient to effect the basic biological process of ligand-induced receptor tyrosine kinase down-regulation, and the less conserved C-terminal sequences are dispensable for this function.

Experimental Procedures

Plasmids—The pAlterMax (Promega, Madison, WI) derivatives pAlterMAX-HA-Cbl, for expression of hemagglutinin (HA) epitope-tagged wild-type human Cbl, and pAlterMAX-HA-Cbl-G306E, for expression of full-length Cbl containing a glycine to glutamic acid amino acid substitution at position 306, have been described (26). Constructs pAlterMAX-HA-Cbl-N, encoding Cbl amino acids 1–357, and pAlterMAX HA-Cbl-N-G306E were generated by subcloning the v-club-containing BamHI fragments from corresponding pSRneo constructs (12, 22) into the pAlterMAX-2 vector. The pAlterMAX-2 vector was derived by site-directed mutagenesis of pAlterMAX by mutagenic primer 5′-GTT CGA-CTC-TAG-AGG-ATC-GAC-GCG-TGA-ATT-CT-3′, which converted the XhoI site to a BamHI site. Constructs encoding green fluorescent protein (GFP) fused at its C terminus to various Cbl proteins were generated by replacing the p53 coding sequences of pCDGFPp53 (38) with BamHI/XhoI fragments derived from products of polymerase chain reaction amplification of pAlterMAX-HA-Cbl or pAlterMAX-HA-Cbl-G306E plasmids. Cbl sequences for amino acids 2–906 were amplified using the primers 5′-CCC-GGA-TCC-GGC-GAC-CTG-GG-AAG-AGC-3′ and 5′-CCC-CGG-GAC-CTG-GTT-TAC-ATG-GGC-AAC-3′. Cbl sequences coding for amino acids 2–357 were amplified using the primers 5′-CCC-GGA-TCC-GGC-GAC-CTG-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-GT-...
leupeptin, pepstatin, antipain, and chymostatin) as described (26). The protein concentration of lysates was determined using the Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard.

Immunoprecipitation and Immunoblotting—The procedures for immunoprecipitation and immunoblotting were previously described (33). Among the various reagents used and the antibodies employed, the relevant figure legends. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA). Immunodetection was performed using horseradish peroxidase-conjugated antibody (A Cappel/Organon Teknika Corp., West Chester, PA) and the Renaissance Western blot Chemiluminescence Reagent Plus kit (NEN Life Science Products). Where indicated, membranes were stripped and reprobed as described previously (33).

For anti-ubiquitin immunoblotting, a modification of a published protocol (46) was used. Following transfer of gel-resolved proteins to a PVDF filter, the membrane was submerged in water and autoclaved for 15 min. The filter was processed for immunodetection as described above, except that the blocking solution contained sodium fluoride (10 mM) and sodium orthovanadate (1 mM), 5% non-fat dry milk rather than 2% gelatin, and the Tween 20 concentration in appropriate solutions was 0.1%.

Flow cell Immunostaining and Fluorescence Microscopy—The procedure for immunostaining was a modification of that previously reported (47). COS-7 cells were seeded onto sterile glass coverslips in 15-cm cell culture dishes. Following attachment, the cells were transfected by the calcium phosphate method, using 15 μg of the appropriate pCD-GFP DNA construct. At 48 h post-transfection, coverslips were harvested in staining buffer and fixed by addition of an equal volume of 3% paraformaldehyde. Flow cytometry, data collection, and analysis were performed on a FACSort machine using CellQuest software (Becton Dickinson, West Chester, PA) and the Renaissance Western blot Chemiluminescence Reagent Plus kit (NEN Life Science Products). Where indicated, membranes were stripped and reprobed as described previously (33).

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Analysis of Receptor Turnover—The turnover of EGF-R in 293 HEK cells expressing endogenous Cbl (mock-transfected) versus those over-expressing HA-Cbl was evaluated by pulse-chase analysis using a modification of the previously reported protocol (48). In brief, cells were transfected in 10-cm tissue culture dishes by the calcium phosphate technique to yield subconfluent cultures at 48–72 h post-transfection. Cells were rinsed with methionine-free DMEM and methionine-starved for 2 h at 37 °C by incubation in methionine-free DMEM supplemented with 0.5% dialyzed FBS (Life Technologies, Inc.). Cells were pulse-labeled by adding 50 μCi/ml EXPRESS labeling mix (NEN Life Science Products) for 40 min at 37 °C. After three washes in DMEM, the cells were incubated in a 0.5% dialyzed FBS and 3 mg/ml l-methionine) to allow newly synthesized EGF-R to undergo post-translational modification, acquire the capacity to bind to ligand, and be transported to the cell surface. One plate from each transfection set was harvested without further EGF stimulation (0 min stimulation). Cells in the remaining plates were incubated in chase medium containing EGF (100 ng/ml) for the indicated times. Lyse preparation, protein quantification, immunoprecipitation, and gel resolution and PVDF membrane immobilization of proteins were performed as described above. The membrane was used first for autoradiography for detection of radiolabeled proteins (BI-O MAX-MR film, Eastman Kodak Co.), and then in anti-EGF-R immunoblotting to confirm the identity of specific radiolabeled proteins as EGF-R. The turnover protocol was performed using a Hewlett-Packard ScanJet 4c™ scanner and Corel Draw™ version 6 software.

RESULTS

Hemagglutinin- and Green Fluorescent Protein-tagged Cbl Proteins Are Functionally Equivalent—We previously showed that Cbl negatively regulates the Syk and PDGF-R-α tyrosine kinases by inducing kinase degradation (26, 27). In the case of Cbl-mediated down-regulation of PDGF-R-α, ligand-dependent ubiquitination of cell surface PDGF-R-α was significantly enhanced when wild-type Cbl was overexpressed (27). Others (28) recently showed that Cbl similarly induces ligand-dependent ubiquitination, cell surface down-regulation, and degradation of the EGF-R. The experiments described here were designed to define the Cbl domains sufficient to effect ligand-dependent EGF-R down-regulation.

We chose to assay down-regulation of cell surface EGF-R using fluorescence-based assays of transiently transfected cells. We generated a series of constructs encoding wild-type and mutant Cbl proteins fused at their N termini to a variant of the green fluorescent protein (49). Since GFP-tagged Cbl proteins were not reported previously, we compared their biochemical properties with those of their hemagglutinin (HA)-Cbl counterparts, which have been shown to associate with activated EGF-R (10, 11, 24, 29–35, 50).

Experiments were conducted using a 293 HEK cell transient transfection system. The expression of endogenous EGF-R in 293 HEK cells is relatively low, making it difficult to detect Cbl-associated receptor (see Fig. 1A, lanes 1 and 2). However, transfection of 293 HEK cells with an EGF-R expression construct resulted in readily detectable levels of receptor (Fig. 1A, compare lanes 1 and 2 with lanes 3–12). Tyrosine phosphorylation of the exogenous EGF-R was strictly EGF-dependent over a broad range of receptor expression levels, indicating that it was appropriately regulated (Fig. 1B, lanes 3–12).

Transient transfection of the HA- or GFP-tagged wild-type Cbl constructs produces Cbl fusion proteins (Fig. 2A) able to associate with ligand-activated EGF-R, as demonstrated by co-precipitation in anti-HA or anti-GFP immunoprecipitates (Fig. 2B). For both the HA-tagged and GFP-tagged Cbl fusion proteins, association with the EGF-R correlated with ligand-dependent tyrosine phosphorylation of both the EGF-R and Cbl proteins (Fig. 2C). A stimulation time course indicated that the levels of both tagged Cbl proteins were maintained during prolonged incubation of cells with EGF (Fig. 2A). How-
transfected EGF-R DNA (ng): 0 25 50 100 250 500 2
EGF stimulation: - + - + - + - + - + - + - + - + - +

Blot: anti-EGF-R
lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

B

Blot: anti-Tyr (P)
lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

C

FIG. 1. Tyrosine phosphorylation of exogenous EGF-R is EGF-dependent over a broad range of protein expression levels. Paired cultures of 293 HEK cells were transiently transfected using graded doses of the EGF-R/pAlterMAX construct or vector alone. The amount of EGF-R DNA transfected per 10-cm dish is indicated. Total DNA (5 μg per dish) and promoter input levels were equalized among transfections using pAlterMAX vector. At 72 h post-transfection, cultures were subjected to serum starvation, followed by incubation without or with EGF for 1 min at the temperature indicated. Cell lysates were prepared as described under “Experimental Procedures.” Equal amounts (50 μg) of lysate protein from each sample were resolved by SDS-PAGE and transferred to a PVDF membrane. The membrane was immunoblotted (A) with anti-EGF-R antibody EGFR 1005, then was stripped and reprobed (B) with anti-Tyr(P) antibody 4G10.

However, the level of Cbl tyrosine phosphorylation decreased over time, as expected (Fig. 2C). Notably, the amount of Cbl-associated EGF-R decreased over time, regardless of the epitope tag present (Fig. 2B).

Next, we compared the biochemical characteristics of wild-type and mutant GFP-Cbl proteins. All GFP-Cbl constructs encoded proteins of the correct size (Fig. 3A) and immunoreactivity (Fig. 3B). Multiple biochemical properties of the various GFP-tagged proteins matched those described for their HA-tagged counterparts (35). First, the GFP-tagged full-length wild-type and G306E mutant Cbl proteins underwent detectable EGF-dependent tyrosine phosphorylation (Fig. 3C, open arrow), but phosphorylation of the G306E mutant (lanes 5 and 6) was significantly less than that of wild-type GFP-Cbl (lanes 2 and 3). Second, full-length wild-type and G306E Cbl proteins, as well as the Cbl-N truncation mutant, associated with EGF-activated EGF-R (Fig. 3E, lanes 2 and 3, 5 and 6, and 8 and 9, respectively). Third, GFP-Cbl-N-G306E, which lacks both the Cbl-C region and a functional TKB domain, failed to associate with activated EGF-R (Fig. 3E, lanes 11 and 12). Taken together, these results indicated that the GFP-tagged Cbl proteins were biochemically comparable to their HA-tagged counterparts and were therefore suitable for further analysis.

Wild-type Cbl, but Not a TKB Domain-inactive Mutant, Enhances Ligand-induced EGF-R Ubiquitination and Turnover—The EGF-R undergoes ubiquitination upon ligand-dependent activation (51); overexpression of wild-type Cbl enhances this effect (28). We tested whether the ligand-induced, Cbl-dependent enhancement of EGF-R ubiquitination requires a functional Cbl TKB domain.

We first confirmed that GFP-tagged Cbl proteins were suitable reagents for this analysis. Transfected 293 HEK cells transiently expressing EGF-R alone or in combination with wild-type HA-Cbl or GFP-Cbl were harvested at various times after EGF stimulation. Immunoblots of anti-EGF-R immunoprecipitates (Fig. 4) showed that, although EGF-R expression and gel motility were similar among the transfectants prior to activation of the cells (Fig. 4A, lanes 2, 9, and 16), stimulation with EGF induced differential effects. Significant smearing of the EGF-R band was apparent in samples derived from HA- or GFP-Cbl overexpressing cells treated with EGF (Fig. 4A, lanes 10–15 and 17–22; also, Fig. 7B, top panel, 3rd and 5th lanes). Immunoblotting with an anti-ubiquitin antibody revealed that the smears contained ubiquitinated EGF-R (Fig. 4B, lanes 10–15 and 17–22; also Fig. 7B, middle panel, lane 5). The highest ubiquitin levels were detected in the upper portions of the EGF-R smears, which contained only a small fraction of the cellular pool of EGF-R (compare Fig. 4, A and B, bracketed regions; also Fig. 7B, bracketed regions). This was consistent with an expected reduction of EGF-R gel mobility upon receptor multiquitination. As observed for HA-Cbl, overexpression of exogenous GFP-Cbl accelerated the apparent turnover of activated EGF-R (Fig. 4A, compare lanes 3–8 with lanes 10–15 and 17–22). Thus, the GFP-Cbl proteins were suitable tools to study ligand-dependent ubiquitination and turnover of EGF-R.

Neither the GFP-Cbl-G306E mutant (Fig. 5B, lanes 2–7), which bears an inactivated TKB domain together with an intact Cbl-C region, nor GFP-Cbl-N (Fig. 5A, lanes 12–17), which bears an intact TKB domain but lacks the Cbl-C region, increased EGF-R ubiquitination or turnover beyond the levels...
observed for expression of EGF-R together with either GFP alone or one of the following GFP-Cbl fusion proteins: GFP-Cbl, GFP-Cbl-G306E, GFP-Cbl-N, or GFP-Cbl-N-G306E. Transfected cells were subjected to the standard EGF stimulation protocol, collected after various times of EGF stimulation, and processed for live cell immunostaining of cell surface EGF-R. The GFP-positive cells were then assayed by flow cytometry for cell surface EGF-R levels. The mean fluorescence intensity (MFI) of specific EGF-R staining was determined, and the MFI values at different times after EGF addition were expressed as percentages of the initial MFI of unstimulated cells.

We first established that binding of the anti-EGF-R antibody to cell surface receptors was not disrupted by incubation of 293 HEK cells with EGF. This was a significant consideration, as the antibody binds an extracellular epitope of EGF-R and has been reported in certain systems to interfere with ligand-receptor interactions and ligand-induced tyrosine kinase activity (52–54). Control experiments showed that our method of sample processing removed cell surface-associated EGF, leaving surface receptors available for binding by the anti-EGF-R antibody (data not shown). Thus, immunostaining could be used to compare surface EGF-R levels of unstimulated and EGF-stimulated 293 HEK cells expressing the various GFP-Cbl proteins.

Incubation of 293 HEK cells with EGF at 37 °C induced tyrosine phosphorylation of exogenous EGF-R (Fig. 1B, compare lanes 3 and 14). However, incubation of a matched culture with EGF at 0 °C induced no detectable EGF-R tyrosine phosphorylation (Fig. 1B, lane 14), despite demonstrable binding of EGF to cell surface receptors (data not shown). This indicated that ligand-induced, activation-associated biochemical changes in the EGF-R do not proceed in this system at 0 °C. Therefore, we considered it unlikely that immunostaining of live cells at 0 °C would have any adverse impact on the subsequent quantification of cell surface EGF-R levels.
transiently transfected using the EGF-R/pAlterMAX construct (0.05 μg). Cultures of 293 HEK cells in 10-cm dishes were transfected using EGF-R together with GFP-Cbl constructs (2 μg). Total DNA was 2.05 μg per dish. Cells were serum-starved and EGF-stimulated as described in Fig. 2. Matched plates for each transfection were harvested at various times following EGF stimulation and were processed for live cell immunostaining and flow cytometry as detailed under “Experimental Procedures.” The EGF-R-specific MFI at each time point is expressed as a percentage of the initial EGF-R-specific MFI of unstimulated cells that were identically transfected. The data shown represent the mean values obtained from three independent experiments, ±S.D. The symbols used are as follows: GFP, closed circles; GFP-Cbl-wt, squares; GFP-Cbl-G306E, triangles; GFP-Cbl-N, ×; and GFP-Cbl-N-G306E, diamonds.

Compared with GFP transfectants, the modulation of surface EGF-R levels in cells expressing GFP-tagged Cbl and Cbl-N was dramatically altered. Overexpression of GFP-Cbl enhanced the down-regulation of surface EGF-R beyond the level observed for control cells (Fig. 6, compare squares and circles). In contrast, the transforming Cbl-N protein drastically reduced down-regulation of cell surface EGF-R, compared with mock-transfected cells (Fig. 6, compare × and circles). The opposite effects of Cbl-N and wild-type Cbl suggest a dominant inhibitory effect of Cbl-N on endogenous Cbl function. These results mirrored those obtained by Yarden and colleagues (28) using wild-type Cbl and Cbl-N in a different assay system, further validating our approach.

Overexpression of the EGF-R non-binding mutant GFP-Cbl-N-G306E (Fig. 3E, lanes 11 and 12) had no significant impact on the kinetics of receptor loss from the cell surface (Fig. 6, compare diamonds and circles). However, overexpression of the EGF-R-binding mutant GFP-Cbl-G306E (Fig. 3E, lanes 5 and 6) retarded surface EGF-R down-regulation at early and late times following EGF addition (Fig. 6, compare triangles and circles). Suppression of EGF-R down-regulation by GFP-Cbl-G306E was reproducibly less than that effected by GFP-Cbl-N. This suggests that the TKB domain-inactivated G306E mutant is a dominant negative inhibitor of surface EGF-R down-regulation by endogenous wild-type Cbl but is functionally less potent than Cbl-N.

Together, these results indicate that Cbl-mediated enhancement of cell surface EGF-R down-regulation requires a functional TKB domain (inactivated in Cbl-G306E; present in Cbl-N) as well as additional sequences in the Cbl-C domain (present in Cbl-G306E; absent from Cbl-N). These structural requirements are identical to those for Cbl-mediated enhancement of EGF-R ubiquitination (Fig. 5, A and B). To identify the Cbl sequences sufficient to enhance EGF-R down-regulation, GFP-tagged C-terminal truncation mutants of Cbl were tested in the FACS-based assay. A protein comprising Cbl amino acids

Cbl Sequences Sufficient for EGF Receptor Down-regulation

Fig. 6. Ligand-dependent down-regulation of cell surface EGF-R by wild-type Cbl but not by mutant Cbl proteins. Replicate cultures of 293 HEK cells in 10-cm dishes were transiently transfected using the EGF-R/pAlterMAX construct (0.05 μg), in combination with the indicated GFP-Cbl constructs (2 μg). Total DNA was 2.05 μg per dish. Cells were serum-starved and EGF-stimulated as described in Fig. 2. Matched plates for each transfection were harvested at various times following EGF stimulation and were processed for live cell immunostaining and flow cytometry as detailed under “Experimental Procedures.” The EGF-R-specific MFI at each time point is expressed as a percentage of the initial EGF-R-specific MFI of unstimulated cells that were identically transfected. The data shown represent the mean values obtained from three independent experiments, ±S.D. The symbols used are as follows: GFP, closed circles; GFP-Cbl-wt, squares; GFP-Cbl-G306E, triangles; GFP-Cbl-N, ×; and GFP-Cbl-N-G306E, diamonds.

Compared with GFP transfectants, the modulation of surface EGF-R levels in cells expressing GFP-tagged Cbl and Cbl-N was dramatically altered. Overexpression of GFP-Cbl enhanced the down-regulation of surface EGF-R beyond the level observed for control cells (Fig. 6, compare squares and circles). In contrast, the transforming Cbl-N protein drastically reduced down-regulation of cell surface EGF-R, compared with mock-transfected cells (Fig. 6, compare × and circles). The opposite effects of Cbl-N and wild-type Cbl suggest a dominant inhibitory effect of Cbl-N on endogenous Cbl function. These results mirrored those obtained by Yarden and colleagues (28) using wild-type Cbl and Cbl-N in a different assay system, further validating our approach.

Overexpression of the EGF-R non-binding mutant GFP-Cbl-N-G306E (Fig. 3E, lanes 11 and 12) had no significant impact on the kinetics of receptor loss from the cell surface (Fig. 6, compare diamonds and circles). However, overexpression of the EGF-R-binding mutant GFP-Cbl-G306E (Fig. 3E, lanes 5 and 6) retarded surface EGF-R down-regulation at early and late times following EGF addition (Fig. 6, compare triangles and circles). Suppression of EGF-R down-regulation by GFP-Cbl-G306E was reproducibly less than that effected by GFP-Cbl-N. This suggests that the TKB domain-inactivated G306E mutant is a dominant negative inhibitor of surface EGF-R down-regulation by endogenous wild-type Cbl but is functionally less potent than Cbl-N.

Together, these results indicate that Cbl-mediated enhancement of cell surface EGF-R down-regulation requires a functional TKB domain (inactivated in Cbl-G306E; present in Cbl-N) as well as additional sequences in the Cbl-C domain (present in Cbl-G306E; absent from Cbl-N). These structural requirements are identical to those for Cbl-mediated enhancement of EGF-R ubiquitination (Fig. 5, A and B). To identify the Cbl sequences sufficient to enhance EGF-R down-regulation, GFP-tagged C-terminal truncation mutants of Cbl were tested in the FACS-based assay. A protein comprising Cbl amino acids
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Fig. 7. Cbl amino acids 1–440 are sufficient to enhance ligand-dependent down-regulation of cell surface EGF-R. Replicate cultures of 293 HEK cells in 10-cm dishes were transiently transfected using the EGF-R/AlterMAX construct (0.1 μg), in combination with the indicated GFP-Cbl constructs (4 μg). Total DNA was 4.1 μg per dish. Cells were serum-starved and EGF stimulated as described in Fig. 2. Matched plates for each transfection were harvested at various times following EGF stimulation and were processed for live cell immunostaining and flow cytometry (A) or cell lysate preparation (B) as detailed under “Experimental Procedures.” A, the EGF-R-specific MFI at each time point is expressed as a percentage of the initial EGF-R-specific MFI of unstimulated cells that were identically transfected. The data shown represent the mean values obtained from three independent experiments, ±S.D. The symbols used are as follows: GFP, closed circles; GFP-Cbl-wt, squares; GFP-Cbl-N, ×; GFP-Cbl-(1–440), triangles. B, where indicated, cells were stimulated with EGF for 10 min. Equal amounts (1500 μg) of lysate protein were immunoprecipitated using anti-EGF-R antibody EGF R528. Immunoprecipitates (I.P.) and corresponding lysates (150 μg) were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were immunoblotted using the antibodies indicated. Brackets in the top and middle panels mark the same region of a single membrane that was sequentially immunoblotted with anti-ubiquitin (Ub) and anti-EGF-R antibodies. The positions of the 175-, 83-, and 62-kDa prestained molecular mass markers are shown to the right.

1–440 fused to GFP was as effective as wild-type GFP-Cbl in enhancing the down-regulation of cell surface EGF-R (Fig. 7A). Furthermore, the 1–440 Cbl truncation mutant was sufficient to enhance ligand-dependent EGF-R ubiquitination (Fig. 7B).

Wild-type Cbl, but Not TKB Domain-inactive Mutant, Enhances Targeting of Activated EGF-R to Vesicles of the Endocytic Pathway—Given that overexpression of Cbl in 293 HEK cells enhanced the down-regulation of activated cell surface EGF-R, we wished to determine the subsequent intracellular fate of the receptor in cells expressing wild-type or mutant Cbl proteins. Previous reports have shown that Cbl and EGF-R colocalize to intracellular vesicular structures upon stimulation of cells with EGF (10, 28, 32). However, the relevance of this compartmentalization to Cbl-mediated EGF-R down-regulation has not been established. Furthermore, the role of the Cbl TKB domain in vesicular colocalization of Cbl and EGF-R is unknown.

We transiently expressed GFP-tagged wild-type or mutant Cbl proteins in COS-7 cells in order to assess the intracellular localization of EGF-R and GFP-tagged Cbl proteins after various periods of EGF stimulation. COS-7 cells were used in these analyses for several reasons. First, their strong substratum attachment allows for reproducible immunostaining on glass coverslips. Second, their relatively low nucleus-to-cytoplasmic ratio facilitates visualization of cytoplasmic structures by microscopy. Third, substantial endogenous EGF-R expression on these cells permits immunodetection of the receptor without expression of exogenous EGF-R.

Examination of GFP-Cbl-transfected COS-7 cells in the absence of EGF stimulation revealed diffuse and reticular green fluorescence that was excluded from the nucleus (Fig. 8B). This was consistent with the primarily cytosolic localization previously reported for endogenous Cbl (10, 32, 55). The EGF-R was predominantly distributed at the surface of unstimulated cells, with minor localization in punctate vesicular structures (Fig. 8A). As soon as 5–10 min after stimulation with EGF (Fig. 8, D and F), the GFP-Cbl fluorescence became focused in small vesicular structures whose size increased with the length of the stimulation period (Fig. 8, H and J). Concurrently, EGF-R accumulated in punctate structures consistent in pattern with endosomes of the coated pit pathway (Fig. 8C) whose role in internalization of EGF-R is established (56). Colocalization of EGF-R and GFP-Cbl in somewhat larger vesicular structures was clearly evident by 10–20 min of stimulation (Fig. 8, E–H). At the 10- and 20-min time points, enhanced down-regulation of cell surface EGF-R in GFP-Cbl expressing cells was obvious, as reflected by significantly reduced overall rhodamine staining on the plasma membrane of transfected cells when compared with adjacent untransfected cells (Fig. 8, E and G). Concomitant with the loss of surface staining, large EGF-R-containing intracellular vesicular structures accumulated in Cbl-transfected cells (Fig. 8G). Relative to adjacent untransfected cells, the size of these vesicles was enhanced. These results suggest that wild-type Cbl overexpression increased targeting of the EGF-R to the endocytic pathway following its down-regulation from the cell surface. These results are in agreement with those reported by Yarden and colleagues (28).

The mutant GFP-Cbl proteins were evaluated in the same system. COS-7 cells expressing the various GFP-Cbl proteins were EGF-stimulated for 30 min, and the intracellular distributions and levels of EGF-R and GFP-Cbl were examined (Fig. 9, A–H). Similar to the distribution of wild-type GFP-Cbl in unstimulated cells, GFP-Cbl-G306E showed a cytosolic distribution and was excluded from the nucleus (Fig. 9D), whereas the GFP-Cbl-N (Fig. 9F) and GFP-Cbl-N-G306E (Fig. 9H) proteins were found in both the nucleus and cytoplasm, as previously reported for untagged Cbl-N (55).

In contrast to EGF-stimulated wild-type Cbl-expressing cells, EGF-stimulated COS-7 cells expressing the GFP-Cbl-
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G306E mutant protein contained few, if any, GFP-positive endocytic vesicles, even at later times of stimulation (Fig. 9D). This result is striking, given that the Cbl-G306E mutant associated well with EGF-R in biochemical assays (Fig. 3C). However, GFP-Cbl-N associated with small EGF-R-positive endocytic vesicles at approximately the same time after EGF stimulation as did wild-type Cbl (Fig. 9F, and data not shown). Notably, the dramatic increase in vesicle size observed with wild-type Cbl at late times after EGF stimulation was not apparent with Cbl-N (compare Fig. 9, B with F). GFP-Cbl-N-G306E remained diffusely distributed and at no time after stimulation localized to endocytic vesicles (Fig. 9H, and data not shown).

The pattern of EGF-R localization in EGF-stimulated cells expressing GFP-Cbl-G306E and GFP-Cbl-N-G306E resembled that of untransfected adjacent cells (Fig. 9, C and G, respectively), demonstrating that TKB domain function is essential for Cbl-mediated enhancement of EGF-R recruitment to vesicles of the endocytic pathway.

Notably, expression of GFP-Cbl-N enhanced cell surface EGF-R staining (Fig. 9E). This qualitative result is consistent with the quantitative results of the live cell immunostaining/flow cytometry experiments (Fig. 6). Importantly, whereas GFP-Cbl and GFP-Cbl-N both colocalized with EGF-R to intracellular vesicular structures for extended periods after EGF stimulation, the two proteins ultimately induced differential effects on EGF-R trafficking. The potential significance of this finding is addressed under “Discussion.”

Wild-type Cbl Enhances the Degradation of EGF-R—To determine the level to which Cbl augmented the turnover of activated EGF-R, a pulse-chase analysis was performed in EGF-R- and HA-Cbl-cotransfected 293 HEK cells. In the absence of Cbl overexpression, stimulation of cells with EGF led to a small but detectable decrease in the labeled pool of EGF-R, consistent with EGF-induced receptor degradation (Fig. 10, open squares). However, the receptor levels plateaued within 90 min and remained constant throughout the rest of the chase period. In contrast, cells in which wild-type Cbl was overexpressed showed substantially enhanced turnover of the labeled pool of EGF-R (Fig. 10, closed squares). Comparison of the two curves between 0 and 90 min of chase indicated that the initial turnover rate for EGF-R was similar in the presence or absence of exogenous Cbl. However, turnover of EGF-R continued in Cbl-overexpressing cells beyond the plateau observed in mock transfectants. These results suggest that Cbl facilitates EGF-R degradation by overcoming a limiting step in the endocytic sorting/lysosomal degradation pathway.

**Discussion**

We report that Cbl-mediated enhancement of EGF-R ubiquitination, cell surface down-regulation, endocytic pathway targeting, and degradation is TKB domain-dependent. Furthermore, this enhancement does not require the proline-rich region, leucine zipper, or tyrosine phosphorylation sites present within the C terminus of Cbl.

Clues to a potential mechanism for Cbl-mediated down-regulation of EGF-R activity were obtained by studying other tyrosine kinases that are negatively regulated by Cbl. Suppres-
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Overexpression of wild-type Cbl enhances ligand-dependent EGF-R degradation. Replicate cultures of 293 HEK cells in 10-cm dishes were transiently transfected using the EGF-R/pAlterMAX construct (0.05 μg) with or without the HA-Cbl construct (2 μg). Total DNA (2.05 μg per dish) and promoter input levels were equalized among transfections using pAlterMAX vector. The 293 HEK cells were used in a pulse/chase protocol, as described under “Analysis of Receptor Turnover” under the “Experimental Procedures.” Equal amounts (400 μg) of protein from the lysates were used to generate immunoprecipitates with anti-EGF-R antibody EGFR 528. The relative amount of radiolabeled EGF-R remaining at each time point is represented as a percentage of the maximal EGF-R signal detected throughout the course of the experiment. The experiment was performed twice; a representative result is shown here. The symbols used are as follows: vector, open squares; HA-Cbl, closed squares.

Our results indicate that at least two Cbl domains are required for efficient colocalization of Cbl and EGF-R binding with the ability of the EGF-R to be endocyotized in response to activating ligand indicates that Cbl is a functional Cbl TKB domain appears to be necessary (the Cbl-G306E mutant showed poor colocalization with EGF-R). However, the TKB domain is not sufficient to effect down-regulation of internalized EGF-R (Cbl-N colocalized with EGF-R in endocytic vesicular structures, yet failed to enhance receptor degradation). Apparently, Cbl-mediated enhancement of EGF-R transit through the endocytic pathway involves a commitment step downstream of Cbl/EGF-R recruitment to endosomes, and it is at this point that the fates of Cbl/EGF-R and Cbl-N/EGF-R complexes diverge. A second Cbl domain is clearly required at this point to route Cbl/EGF-R complexes toward the lysosomal degradation pathway.

In conjunction with the TKB domain, Cbl amino acids 358–440 are sufficient to effect enhanced degradation of internalized EGF-R. The latter region of Cbl encompasses the evolutionarily conserved RING finger domain that is present in all Cbl homologues that suppress EGF-R signaling in vivo (7, 9–11), and that was shown recently to play a role in EGF-R down-regulation (60). Ligand-activated, internalized EGF-R is either targeted to the lysosome or recycled back to the cell surface, and the point of divergence for these routing pathways lies within the multivesicular bodies (61–65). Further studies will determine whether the Cbl TKB and 358–440 domains effect enhanced receptor degradation at this stage of the EGF-R trafficking pathway.

One remarkable aspect of data presented here and elsewhere (28) is that overexpressed wild-type Cbl enhanced ligand-dependent EGF-R down-regulation beyond the level observed for cells expressing only the endogenous Cbl. These results raise two possibilities as follows: either Cbl is a limiting factor required to route EGF-R/wild-type Cbl complexes into the endosomal/lysosomal pathway for protein degradation, or overexpression of Cbl inactivates a repressor that normally limits entry of EGF-R into the endosomal/lysosomal degradation pathway. Both interpretations invoke the existence of an EGF-R endocytosis pathway that is saturable, as has been demonstrated (66). Although our current results do not favor one interpretation over the other, they implicate Cbl as a limiting factor that could set the saturation level of the EGF-R down-regulation pathway.

A vast body of research into the EGF-R domains required for ligand-dependent receptor endocytosis has been generated, but no single domain has been identified as the ultimate effector site sufficient for receptor down-regulation (56, 64, 67–81). It is unclear at present whether there is a link between EGF-R sequences critical for receptor endocytosis and their potential interaction with or regulation by Cbl. However, the attractiveness of Cbl as a candidate regulator of EGF-R endocytosis is enhanced by the demonstration that the tyrosine kinase activity of EGF-R is critical for its recruitment into clathrin-coated pits of the endocytic pathway (82). Interestingly, removal of the autophosphorylation sites from the EGF-R tail does not abrogate the requirement of receptor kinase activity for recruitment of EGF-R into coated pits. Lamaze and Schmid (82) have proposed that trans-phosphorylation of a cytosolic factor by the EGF-R kinase and has been shown (Ref. 28 and this study) to affect down-regulation and trafficking of activated EGF-R. Further investigations will delineate the contributions made by Eps15, EAST, and phospholipase C γ-1, other EGF-R substrates implicated in regulating receptor endocytosis (83–88).

Cumulative evidence from our laboratory indicates that Cbl down-regulates tyrosine kinases by programming them for ubiquitination and degradation. Similar results have been ob-
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...in studies of the PDGF-R-β and colony-stimulating factor-1 receptor (89, 90). Ubiquitination of cell surface receptors facilitates their down-regulation via either of two mechanisms: proteinase-mediated degradation or enhanced endocytosis and degradation in the lysosomes (91–95). Future studies will determine whether Cbl-enhanced ubiquitination of EGF-R is a signal for receptor internalization and degradation.

The Cbl sequences sufficient to enhance EGF-R ubiquitination and down-regulation (amino acids 1–440) are those conserved among all members of the Cbl family of proteins. This raises the following question: what are the functions of the less conserved Cbl domains that are dispensable for EGF-R down-regulation? The C-terminal domains may be important for regulating tyrosine kinases other than EGF-R. Alternatively, they may play critical roles during development or in specific cell types. Further studies are clearly indicated to assign functions to the C-terminal proline-rich sequences, leucine zipper, and tyrosine phosphorylation sites.

It is noteworthy that the Cbl sequences dispensable for EGF-R down-regulation include the proline-rich region that directly binds the SH3 domain of the adapter protein Grb2 (reviewed in Ref. 1). Grb2 binds to EGF-R via its SH2 domain, and the interaction is critical for EGF-dependent receptor endocytosis (96). This finding raised the possibility that Grb2 might act as an adapter for indirect association of EGF-R with Cbl via interaction of the Grb2 SH3 domain and the Cbl proline-rich region and that the indirect association might contribute to Cbl-mediated EGF-R down-regulation. This hypothesis is incompatible with the results reported here, as deletion of the proline-rich region of Cbl had no discernible impact on Cbl-mediated enhancement of EGF-R down-regulation.

In summary, the studies presented here, together with the recent results of Levkovitz and colleagues (28), lead us to conclude that the proto-oncoprotein Cbl negatively regulates the EGF-R by enhancing ligand-dependent receptor ubiquitination, trafficking through the endocytic pathway, and degradation. Overexpression of wild-type Cbl bypasses a limiting step in the saturable pathway for EGF-R down-regulation, resulting in enhanced receptor turnover. We show here that Cbl-mediated enhancement of EGF-R down-regulation is mediated by an intact Cbl TKD domain in conjunction with sequences encompassed by Cbl amino acids 358–440. Our results define structural domains that are required or dispensable for Cbl, which is evolutionarily conserved both in sequence and in function, to effect biologically relevant suppression of ligand-dependent EGF-R signaling.

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