CIN85 Participates in Cbl-b-mediated Down-regulation of Receptor Tyrosine Kinases*

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The Cbl family of ubiquitin ligases in mammals contains three members, Cbl, Cbl-b, and Cbl-3, that are involved in down-regulation of receptor tyrosine kinases (RTKs) by mediating receptor ubiquitination and degradation. More recently, a novel pathway has been identified whereby Cbl promotes internalization of EGF receptor via a CIN85/endophilin pathway that is functionally separable from the ubiquitin ligase activity of Cbl (1). Here we show that Cbl-b, but not Cbl-3, utilize the same mechanism to down-regulate multiple RTKs. CIN85 was shown to bind to the minimal binding domain identified in the carboxyl terminus of Cbl-b. Ligand-induced phosphorylation of Cbl-b further increased their interactions and led to a rapid and sustained recruitment of CIN85 in the complex with EGF or PDGF receptors. Inhibition of binding between CIN85 and Cbl-b was sufficient to impair Cbl-b-mediated internalization of EGF receptors, while being dispensable for Cbl-b-directed polyubiquitination of EGF receptors. Moreover, CIN85 and Cbl/Cbl-b were constitutively associated with activated PDGF, EGF, or c-Kit receptors in several tumor cell lines. Our data reveal a common pathway utilized by Cbl and Cbl-b that may have an important and redundant function in negative regulation of ligand-activated as well as oncogenically activated RTKs in vivo.

Growth-factor binding to receptor tyrosine kinases (RTKs) leads to their activation and subsequent down-regulation through a rapid removal from the cell surface. Recruitment of EGF receptors to clathrin-coated pits as well as their sorting in early endosomes and multivesicular bodies requires kinase activity of the receptors, their ubiquitination, and interactions with multiple regulatory components of endocytic machinery (2, 3). Recent studies have shown that Cbl-mediated ubiquitination of receptors for EGF, PDGF, and CSF-1 is important for targeting these receptors toward the lysosomal degradation pathway (4–6).

The mammalian Cbl protein family consists of three members: Cbl, Cbl-b, and Cbl-3, all having a highly conserved amino-terminal part composed of a tyrosine kinase-binding module and a ring-finger domain (7). This part of Cbl is able to recruit ubiquitin-conjugating enzymes in the complex with activated tyrosine kinase receptors, thus enabling ubiquitination of the receptor molecules (8, 9). However, the carboxyl termini of Cbl proteins are more diversified. Cbl-3 contains only a short polyproline domain in its carboxyl terminus, whereas Cbl and Cbl-b have long proline-rich domains and additional distal parts containing an acidic box and a leucine-zipper (LZ) domain (10). The distal carboxyl-terminal tails of Cbl and Cbl-b contain several polyproline motifs scattered among tyrosine residues that are phosphorylated in vivo after growth-factor stimulation (11). Binding of multiple signaling proteins containing SH2 and SH3 domains to this part of Cbl is regulated by tyrosine phosphorylation (10). Carboxyl-terminal interactions are involved in the control of cell type-specific functions of Cbl, such as regulation of glucose uptake, osteoclast activation, and bone remodeling, as well as cell spreading and migration (12–14).

Several recent reports have also implicated the carboxyl terminus of Cbl in the control of endocytosis of RTKs. The major mechanism of Cbl recruitment to activated EGF receptors involves binding of the SH2 domain of Cbl to the autophosphorylated tyrosine 1045 of EGF receptor (15). An alternative pathway recently discovered by using EGFR-Y1045F mutant was showed to employ the Grb2 adaptor protein, which acts as an intermediate between Cbl and the receptor (16). In addition, binding of SH3 domain-containing protein CIN85 to the distal carboxyl terminus of Cbl was shown to regulate EGF and c-Met receptor endocytosis in mammalian cells (1, 17). CIN85 (Cbl-interacting protein of 85 kDa) is a ubiquitously expressed adaptor protein with three SH3 domains at the amino terminus and a proline-rich region and a coiled-coil domain in the carboxyl terminus. This multi-domain protein binds also to the adaptor proteins Grb2, Crk, and p130Cas and can form larger protein complexes after oligomerization mediated by coiled-coil domain (18). CIN85 was also cloned as Ruk (regulator of ubiquitous kinase) and SETA (SH3 domain-containing gene expressed in tumorigenic astrocytes) and was shown to regulate cell-survival signaling pathways in neuronal and glial cells (19, 20).

Here we describe how Cbl-b mediates down-regulation of activated RTKs, a pathway that depends on binding of CIN85 to an unconventional motif in the distal carboxyl terminus of Cbl-b. CIN85/Cbl-b interaction was critical for a rapid and sustained recruitment of CIN85 in the complex with active EGF and PDGF receptors. In addition, CIN85 and Cbl-b were constitutively associated with activated PDGF, EGF, or c-Kit receptors in dermatofibrosarcoma protuberans (DFSP), A431 breast cancer, and HMC-1 mastocytoma tumor cell lines, respectively. This indicates that Cbl-b and CIN85 participate both in ligand-dependent down-regulation of RTKs and in internalization of constitutively active RTKs found in tumors.
EXPERIMENTAL PROCEDURES

Products and Cloning—EGF was purchased from Intergen; human recombinant 125I-EGF was from Amersham Biosciences. Antibodies recognizing Cbl-b (C2), phosphotyrosine (PY99), and autophosphorylated EGF receptor (phosphotyrosine 1173) were from Santa Cruz Biotechnology, mouse anti-RA from Roche, and mouse anti-FLAG M2 and M5 antibodies from Sigma. Rabbit polyclonal (RR2) as well as mouse monoclonal (109) anti-EGF receptor antibodies were provided by Joseph Schlessinger. Rabbit polyclonal antibodies recognizing casky terminus of phosphotyrosine 1099 of PDGF receptor receptor were provided by Carina Hellberg and Arne Ostman, rabbit polyclonal antibody against c-KIT by Lars Ronnstrand. Details of the rabbit polyclonal sera raised against RING finger of Cbl and Cbl-b (RF) and carboxy-terminal and 477 peptides of CIN85 were described previously (1). Constructs of CIN85 and FLAG-tagged ubiquitin were described previously (1). HA-tagged deletion forms (N1/2 and C2/3) and full size Cbl-b (alternatively spliced form, see Ref. 21) as well as the long form of Cbl-3 in pCEFL were provided by Stanley Lipkowitz. Carboxy-terminal deletions of Cbl-b were created by introducing stop codons at the indicated positions.

Yeast Two-hybrid System Screening—The screening procedure was performed as described in GAL4-based Matchmaker two-hybrid system manual (Clontech), using Y190 yeast clone, pYTH9 GAL4-DNA binding domain vector with full size CIN85 as a bait, and human thymus cDNA library. The yeast retransformation and filter lift assays were performed on selective–Leu–Trp–His agar plates.

Cell Culture and Transfections—CHO, NIH3T3, and HeLa cells were purchased from ATCC. CHO cells with stable expression of EGF receptor were provided by Janie Borst and Anneemieke de Melker. DMSO line was provided by Tobias Sjoblom and Arne Ostman (22). NIH cells with stable expression of EGF receptor were provided by Pier Paolo De Fiore and mast cell leukemia cell lines HMC-1.1 and 1.2 by Gunnar Nilsson. Expression of EGF receptor in CHO clone was maintained by presence of G480 (2.4 mg/ml) in the culture medium. Cells were transfected with LipofectAMINE reagent (Invitrogen) following manufacturer’s instructions. 30 h after transfection, cells were starved for an additional 12 h and stimulated with 100 ng/ml EGF for the times indicated. Cells were lysed in ice-cold 1% Triton X-100 lysis buffer (pH 7.4, 50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol) containing a mixture of protease and phosphatase inhibitors. The lysates were cleared by centrifugation at 13,000 rpm for 20 min at 4 °C.

Immunoprecipitation and Blotting—Lysates with adjusted protein concentration (Bradford assay, Bio-Rad) were incubated with antibodies for 2 h at 4 °C. The following antibodies were used: rabbit polyclonal anti-HA, rabbit polyclonal anti-CIN85 (CT), goat polyclonal anti-Cbl-b, mouse monoclonal anti-FLAG (M2), rabbit polyclonal anti-EGFR (RR2), rabbit polyclonal anti-RING finger Cbl (RF). Immunocomplexes were precipitated after a 1-h incubation with protein A agarose beads. After washing in cold lysis buffer, the complexes were resuspended in Laemmli sample buffer (Bio-Rad), boiled, and resolved by SDS-PAGE.

Immunofluorescence Studies—Cells were seeded on collagen-coated cover slips for 20 h, transfected with indicated plasmids using LipofectAMINE reagent for 24 h, and starved for an additional 12 h. Cells were left untreated or were stimulated with EGF (100 ng/ml) for 10 min at 37 °C and fixed in 4% paraformaldehyde in phosphate-buffered saline for 20 min, treated with 50 mM NH4Cl for 10 min, and permeabilized with 0.2% Triton X-100 for 10 min. After blocking for 1 h in 4% fetal calf serum in phosphate-buffered saline with 2% fetal calf serum, for 90 min, and secondary antibodies were conjugated with fluorescein for 60 min. The preparations were mounted using Fluoromount G, and the images were taken with a Zeiss Axiosplan 2 microscope.

Ligand Internalization Assays—Ligand internalization assays were performed as described (4) with modifications as described in Ref. 1. Briefly, cell monolayers of transfected CHO cells were incubated for 1 h at 4 °C with 125I-labeled EGF, washed twice with binding buffers, and incubated at 37 °C for the time intervals indicated. Cells were transferred on ice and washed with either cold binding buffer or mild acidic buffer to remove surface-bound radiolabeled EGF. The remaining radioactivity in cells was quantified after cell lysis. Each point was measured in quadruplicate and expressed as a percentage (average ± S.D.) of internalized versus total cell-associated radioactive EGF.

EGF Receptor Ubiquitination Assays—HEK293T cells were transfected with cDNA for EGF receptor and flag-tagged ubiquitin together with HA-tagged Cbl-b constructs (wild type) and CIN85 constructs: full size, CIN85–3SH3 or CIN85–PCe in FLAG-pcDNA3, as indicated. Cells were treated with EGF (50 ng/ml) for 10 min at 37 °C and immunoprecipitates of EGFR were blotted with anti-FLAG (M5), and the same membranes were re-probed with anti-EGFR (RK2) antibodies. For each experiment, the levels of transfected Cbl-b and CIN85 constructs were analyzed in cell lysates.

RESULTS

CIN85 Binds to the Distal Carboxy Terminal of Cbl-b and Cbl, but Not Cbl–3—We performed a yeast two-hybrid screen to search for binding partners of CIN85. Screens of human thymus cDNA library with the full size CIN85 gave rise to 25 double-positive clones, two of which encoded the carboxyl terminal of Cbl-b containing the last 124 amino acids. Yeast clones expressing either three SH3 domains of CIN85 (3SH3), a proline-rich region with coiled-coil domain (PCc), or the full size CIN85 were transformed with the carboxyl-terminal part of Cbl-b, and interactions were checked by the ability of clones to grow on selective agars and by filter-lift assays. As shown in Fig. 1A, the 3SH3 domain, but not PCc domain of CIN85 bound to the carboxyl-terminal part of Cbl-b in yeast cells. Similarly, the three SH3 domains, and not the PCc domain, of CIN85 were sufficient for binding to wild-type Cbl-b expressed in mammalian cells (Fig. 1B).

To further analyze Cbl-b domains required for association with CIN85, we co-transfected FLAG-CIN85 together with constructs of Cbl-b (schematically shown in Fig. 1E) in HEK293T cells. Full size Cbl-b, Cbl-b without leucine zipper domain (A927), or Cbl-b without SH2 domain (C2/3) precipitated equal amounts of FLAG-tagged CIN85, whereas Cbl-b constructs with deletions of the distal carboxyl 90 and 190 amino acids (A891 and Δ751) or of the entire carboxyl terminus (N1/2) did not bind to CIN85 (Fig. 1C). These data indicate a minimal binding domain (containing 36 amino acids; 891–927) present in the distal carboxyl terminus of Cbl-b that is critical for binding to CIN85 in mammalian cells. This part of Cbl-b contains three PXXP motifs, known as consensus binding sites for SH3 domains (23). We have mutated the first proline in these PXXP motifs, each separately or all of them together, and showed that neither mutation abrogated binding of CIN85 to Cbl-b (data not shown), suggesting that CIN85 binding to Cbl-b may be mediated by PXXP-independent motifs present in the distal carboxyl terminus of Cbl-b.

Because it was previously reported that CIN85 interacts with all Cbl family members including Cbl, Cbl-b, and Cbl-3 in an in vitro assay (24), we further tested the ability of Cbl-3 to bind to CIN85 in mammalian cells. Although Cbl and Cbl-b readily co-precipitated CIN85, Cbl-3 failed to interact with CIN85 when co-expressed in 293T cells (Fig. 1D). In addition, Cbl-3 did not associate with CIN85 in glutathione S-transferase binding assays and did not colocalize with CIN85 in transfected NIH-EGFR cells (data not shown). Although showing significant similarities in the amino terminus, the carboxyl terminus of Cbl-3 contains only a short proline-rich region and completely lacks the distal part found in Cbl or Cbl-b proteins (25). Taken together with our previous observations (1), these results indicate that CIN85 binds to the distal C termini of Cbl and Cbl-b, but not to Cbl-3, in mammalian cells.

Association of Cbl-b and CIN85 Is Enhanced After EGF and PDGF Stimulation—Previous studies showed that tyrosine phosphorylation of Cbl increases binding of the SH3 domains of CIN85 (1, 26). We therefore tested whether EGF stimulation that leads to strong tyrosine phosphorylation of Cbl affects its interactions with CIN85. HEK293T cells co-expressing EGF receptor, HA-Cbl-b, and FLAG-tagged CIN85 or CIN85–3SH3 constructs were stimulated with EGF, lysed, and subjected to immunoprecipitation with anti-FLAG antibodies. Binding between Cbl-b and CIN85 or CIN85–3SH3 was observed in unstimulated cells, and was significantly increased after EGF
stimulation (Fig. 2A). The basal level of the complex formed in unstimulated cells was most likely caused by activation of EGF receptor kinase due to receptor overexpression.

We next tested whether growth factor stimulation leads to a complex formation between endogenous CIN85, Cbl-b and EGF or PDGF receptors in several cell lines. EGF- or PDGF-stimulated cells were lysed and subjected to immunoprecipitation with antibodies against CIN85 or Cbl-b and the protein complexes were analyzed by immunoblotting with indicated antibodies. A ligand-inducible complex between endogenous CIN85, Cbl-b, and activated EGF receptor was detected in HeLa and NIH3T3-EGFR cells (Fig. 2B) and similarly with PDGF receptors in NIH3T3 cells (Fig. 2C). CIN85 was also found constitutively bound to endophilins (Fig. 2B, left panel) as previously described (1, 17). The complex between CIN85 and activated receptors was detected as early as 5 min and was sustained up to 30 min after PDGF stimulation (Fig. 2D). This result indicates that CIN85 and Cbl-b are rapidly associated with activated receptors and remain anchored in receptor complexes following the receptor endocytosis in the cell. That observation was further tested by analysis of subcellular localization of endogenous CIN85 and Cbl-b in CHO-EGFR cells. In serum-starved cells, CIN85 and Cbl-b were diffusely distributed in cytoplasm, whereas in EGF-treated cells a significant proportion of Cbl-b and CIN85 were found in endocytic vesicles containing clustered receptors (Fig. 3). This is consistent with previous reports showing that Cbl associates with activated EGF receptors at the cell membrane and traffics together with receptor complexes along the endocytic pathway toward lysosome (27), and that CIN85 and Cbl co-localize with activated EGF receptors in endocytic vesicles of CHO cells (1).

Dominant Interfering Forms of CIN85 Block Cbl-b-mediated EGF Receptor Down-regulation—To test the functional significance of CIN85 in regulating EGF receptor endocytosis, we used a dominant interfering form of CIN85 containing the three SH3 domains (CIN85-5SH3) (1) in ligand internalization assays. Expression of Cbl-b together with EGF receptors led to a significant increase in the rate of EGF internalization when compared with cells transfected with EGF receptor alone (Fig. 4A). Cbl-b-accelerated internalization of EGF receptors was blocked by expression of CIN85-3SH3 but not by CIN85 (Fig. 4A). A comparative assay in CHO cells transfected with either
of the three members of the Cbl family showed that Cbl and Cbl-b are associated with oncogenic RTKs and could thus play

...the formation of receptor-containing vesicles upon EGF stimulation was inhibited (Fig. 4C, left panel). Expression of wild-type CIN85 did not lead to inhibition of EGF receptor endocytosis (Fig. 4C, right panel). Interestingly, we have observed that overexpression of CIN85 caused formation of multiple cytoplasmic vesicles that also contained endogenous Cbl-b and Cbl, which further indicates the importance of CIN85 in regulation of the endocytic pathway (Fig. 4C, data not shown, and Ref. 18). Similar observations were also reported for CMS, CIN85-related adaptor protein, which is involved in regulation of the actin cytoskeleton via its Pcc domain (28) and Hrs, a protein with coiled-coil and proline-rich domains, which is found in early endosomes and is involved in EGF receptor down-regulation (29).

Because ubiquitination of receptors was implicated as a signal for internalization (4), we tested whether CIN85 binding to Cbl-b could influence Cbl-b-mediated EGF receptor ubiquitination. Data from transient expression in HEK293T cells showed that activated EGF receptors are ubiquitinated by Cbl-b to the same extent in the absence and in the presence of CIN85 or interfering forms of CIN85 (Fig. 4D, left panel). Moreover, Cbl-b N1/2, which associates with receptor through SH2 domain but is unable to bind to CIN85 (Fig. 1C), facilitated ubiquitination of EGF receptors to similar level as wild-type Cbl-b (Fig. 4D, right panel). Taken together, these results suggested that CIN85 binding to Cbl-b is important for EGF receptor internalization, although it has no effect on Cbl-b-induced receptor ubiquitination. Similarly, cells expressing mutated dynamin K44A showed impaired EGF receptor internalization, whereas EGF receptor poly-ubiquitination was intact (30).

Association of Cbl Proteins and CIN85 with Constitutively Active RTKs in Tumor Cell Lines—PDGF and EGF receptors are expressed in many human tumors, and constitutive activation of their tyrosine kinase domains was shown to contribute to tumor development. For example, autocrine secretion of PDGF is critical for constitutive activation of PDGF receptors in cells isolated from several DFSP patients (22), whereas high overexpression of EGF receptors in breast cancer cell line A431 leads to permanently active receptors and thus contributes to oncogenic transformation (31). In these cell lines, CIN85 and Cbl-b readily co-precipitated PDGF or EGF receptors, and their interactions were only minimally up-regulated by the addition of EGF or PDGF to the medium (Fig. 5, A and B), indicating that CIN85 and Cbl-b constitutively associate with activated PDGF and EGF receptors in human tumor cell lines. Additionally, Cbl-b and CIN85 were co-localized with PDGF receptors in endocytic vesicles of DFSP cells without external addition of PDGF (data not shown).

Gain-of-function point mutations of the c-Kit gene, which encodes a receptor for stem cell factor have been isolated from mast cell tumors and gastrointestinal stromal tumors. A c-Kit mutation in the tyrosine kinase domain (D816V) is commonly found in human mastocytomas, whereas a mutation in the juxtamembrane domain (V560G) is associated with gastrointestinal stromal tumors (32–34). We tested whether CIN85 and Cbl proteins interact with mutant c-Kit receptors found in the human mast cell leukemia cell line HMC-1.1, expressing the V560G mutant, and HMC-1.2, expressing c-Kit receptor with both V560G and D816V mutations. As shown in Fig. 5C, CIN85 and Cbl/Cbl-b were found in the constitutive complexes with oncogenic forms of c-Kit expressed in these cells with only slight increase of complex formation after stimulation with stem cell factor. This suggests that CIN85 together with Cbl/Cbl-b are associated with oncogenic RTKs and could thus play
an important role in receptor down-regulation in transformed cells.

**DISCUSSION**

Dynamic protein interactions and formation of RTK-associated complexes are involved in regulation of multiple steps during receptor internalization and endosomal sorting for recycling or degradation (2, 35). Cbl proteins play a critical role in these processes, by acting as scaffolding molecules and ubiquitin ligases that remain associated with active receptors throughout the endocytic compartments (27). In this report we demonstrate an important role for the adaptor protein CIN85 in mediating Cbl-b-induced down-regulation of activated receptor tyrosine kinases (Fig. 6). After ligand binding, RTKs become tyrosine-phosphorylated and bind to and phosphorylate Cbl-b, which in turn directs ubiquitination of activated receptors (36, 37). Phosphorylated Cbl-b can also recruit CIN85/endophilin complexes in the vicinity of internalizing receptors (Fig. 2B), whereby endophilins may control clathrin-coated vesicle formation during RTK endocytosis (1, 17). Therefore, Cbl-b appears to promote receptor down-regulation via two mechanisms, one dependent on the amino-terminal part of Cbl-b that facilitates receptor ubiquitination, and the other mediated by the carboxyl terminus of Cbl-b that is responsible for binding to CIN85. Receptor ubiquitination is essential for both receptor internalization as well as receptor sorting for lysosomal destruction (30, 38), and all members of the Cbl family (Cbl, Cbl-b, and Cbl-3) were shown to bind to and ubiquitinate activated EGF receptors (15). On the other hand, the CIN85-dependent pathway is essential for receptor internalization, although its function is not directly involved in the control of receptor ubiquitination (Fig. 4, A, C, and D). The importance of this pathway has been demonstrated in the down-regulation of EGF and HGF receptors (1, 17). Here we demonstrate that the CIN85 pathway is specific for Cbl and Cbl-b proteins, but not Cbl-3 (Fig. 1D). Taken together, these data suggest a common CIN85-dependent mechanism by which Cbl and Cbl-b negatively regulate RTKs. Indeed, the functional redundancy for Cbl and Cbl-b is indicated by embryonic lethality of double
Cbl\(^{-/-}\) Cbl-b\(^{-/-}\) mice (39), whereas mice deficient for either Cbl or Cbl-b are viable and show defects in distinct populations of T cells (40, 41).

In addition, CIN85 and Cbl/Cbl-b are associated with constitutively active EGFR, PDGF, and c-Kit receptors in several tumor cell lines, indicating that this mechanism is also involved in down-regulation of oncogenically activated receptor tyrosine kinases (Fig. 5 and 6). The complex formation seems to depend on receptor kinase activity, and all tumor cell lines used express constitutively active, phosphorylated forms of receptors (Fig. 5A) (22, 32, 33, 42). Hyperactivation of RTKs can result from autocrine production of a ligand, overexpression of receptors, or the presence of constitutively active receptor mutants. We addressed these three mechanisms in the respective cell models: dermatofibrosarcoma protuberans, carcinoma A431, and mastocytoma HMC-1.1 and 1.2, and in all cases we found constitutively present receptor-Cbl/Cbl-b-CIN85 complexes (Fig. 5). Whereas in normal cells the signal triggered by ligand-activated RTKs is terminated via Cbl/CIN85-dependent degradation (1), receptors in malignant cells seem to escape down-regulatory mechanisms despite their association with Cbl/Cbl-b and CIN85 and co-localization in endocytic pathways (Fig. 5 and data not shown). Possible mechanisms for this effect may include enhanced receptor recycling, increased synthesis of new active receptors and saturation of endogenous endocytic machinery, which all contribute to the increase in oncogenic potential (2, 43, 44). It is also possible that a proportion of oncogenic receptors is re-localized to intracellular membrane pools from the plasma membrane and so becomes inaccessible for binding of negative regulators. Interestingly, therapeutic treatment of tumors is able to shift that equilibrium by enhancing the degradative path. Cbl was recently shown to mediate the tumor-suppressing effects of anti-ErbB-2 antibodies following receptor croslinking at the plasma membrane and more efficient receptor degradation (45).

The interaction between CIN85 and Cbl/Cbl-b is based on binding of the SH3 domains of CIN85 to distal carboxyl termini of Cbl/Cbl-b. There are two interesting features in the nature of this interaction. First, high affinity binding to Cbl requires multiple SH3 domains of CIN85 (1), and second, their association is regulated by tyrosine phosphorylation of Cbl/Cbl-b (Fig. 2: Ref. 1). The association between CIN85 and the carboxyl terminus of Cbl-b in yeast cells is present even without detectable tyrosine phosphorylation (Fig. 1A). However, in mammalian cells the complex formation between endogenous Cbl-b and CIN85 is highly increased upon ligand-induced phosphorylation of Cbl-b (Fig. 2, B and C). Consistent with these data, CIN85 homologous adaptor protein CMS was shown to bind to the distal proline-rich region of Cbl in a phosphotyrosine-dependent manner (28). It appears that phosphorylation of Cbl/Cbl-b is not directly involved in binding to CIN85 or CMS, but has a regulatory role that promotes conformational change in the carboxyl terminus of Cbl and thus allows CIN85/CMS to bind with higher affinity to polyproline motifs in the distal tail of Cbl proteins. Taken together, tyrosine phosphorylation of Cbl/Cbl-b may control specificity and avidity of SH3 domain-
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containing proteins and thus regulate the dynamics of their interactions. In addition, we identified a minimal binding domain encompassing amino acids 891–927 in the distal carboxyl terminus of Cbl-b that is necessary for efficient binding of CIN85 (Fig. 1C). This CIN85-binding region seems to be particularly conserved between Cbl and Cbl-b, and significant homology is absent in Cbl-3 sequence. Interestingly, CIN85 binding to Cbl-b appears to be mediated via PXXP-independent motifs, since mutation of all PXXP sequences present in the minimal binding domain of the carboxyl terminus of Cbl-b did not abrogate CIN85 binding.

Recent reports have provided evidence for a general mechanism that employs CIN85/endophilin complexes and Cbl/Cbl-b proteins in internalization of numerous tyrosine kinase receptors (1, 17). However, the molecular mechanisms by which CIN85/Cbl and CIN85/Cbl-b complexes control subsequent steps of RTKs endocytosis remain unknown. An additional interesting feature of both Cbl and CIN85 is that they can oligomerize via their leucine zipper or coiled-coil domains, respectively (18, 46). It is possible that an increase in local concentrations of CIN85-Cbl/Cbl-b around internalized RTKs provides spatial coordination for interactions with multiple endocytic proteins. These interactions could in turn control endosomal sorting and targeting of activated RTKs for lysosomal degradation. Taken together our findings suggest that Cbl/Cbl-b and CIN85 play redundant roles in regulating multiple steps in down-regulation of activated tyrosine kinase receptors.

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Note Added in Proof—Recent findings by Haglund et al. (Haglund, K., Shimokawa, N., Szynkiewicz, I., and Dikic, I. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 12191–12196) have demonstrated that ligand-induced binding of CIN85 to Cbl-b is important for Cbl-b-mediated monoubiquitination of CIN85 and their degradation together with EGF receptors in the lysosome.

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