CIN85 participates in Cbl-b-mediated downregulation of receptor tyrosine kinases

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Running title: CIN85 and Cbl-b regulate endocytosis of RTKs

The abbreviations used are:
CIN85, Cbl interacting protein of 85 kDa; DMEM, Dulbecco's modified Eagle's medium; DFSP, dermatofibrosarcoma protuberans; EGF, epidermal growth factor; GIST, gastrointestinal stromal tumor; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet derived growth factor; RTKs, receptor tyrosine kinases; SCF, stem cell factor; SH, Src homology; TCL, total cell lysate.
Summary

The Cbl family of ubiquitin ligases in mammals contains three members Cbl, Cbl-b and Cbl-3 involved in downregulation 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 downregulate 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 e-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- as well as oncogenically-activated RTKs in vivo.
Introduction

Growth factor binding to receptor tyrosine kinases (RTKs) leads to their activation and subsequent downregulation 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 towards 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, while Cbl and Cbl-b have long proline rich domains and additional distal parts containing an acidic box and a leucine-zipper (LZ) domains (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, osteoclasts 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 was recently discovered by using EGFR-Y1045F mutant, and 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 receptors 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 downregulation of activated RTKs, a pathway that is dependent 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 downregulation of RTKs and in internalization of constitutively active RTKs found in tumors.
**Experimental procedures**

**Products and cloning**

EGF was purchased from Intergen; human recombinant $^{125}$I-EGF was from Amersham Pharmacia Biotech. Antibodies recognizing Cbl-b (C-20), phosphotyrosine (PY99) and autophosphorylated EGF receptor (phosphotyrosine 1173) were from Santa Cruz, mouse anti-HA from Roche and mouse anti-FLAG M2 and M5 antibodies from Sigma. Rabbit polyclonal (RK2) as well as mouse monoclonal (108) anti-EGF receptor antibodies were provided by Joseph Schlessinger. Rabbit polyclonal antibodies recognizing C-terminus or phosphotyrosine 1009 of PDGF receptor were provided by Carina Hellberg and Arne Ostman, rabbit polyclonal antibody against c-Kit by Lars Ronnstrand. Details about the rabbit polyclonal sera raised against RING finger of Cbl and Cbl-b (RF) and C-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 (alternative splicing form, see (21)) as well as the long form of Cbl-3 in pCEFL were provided by Stanley Lipkowitz. Carboxyl 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 Matchmarker 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 Jannie Borst and Annemieke de Melker. Cell line established from dermatofibrosarcoma protuberans 149333 tumor (DFSP) was provided by
Tobias Sjoblom and Arne Ostman (22). NIH cells with stable expression of EGFR 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 (Gibco) following manufacturer’s instructions. 30 hours after transfection cells were starved for additional 12 hours and stimulated with 100 ng/ml EGF for indicated times. 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 coctail of protease and phosphatase inhibitors. The lysates were cleared by centrifugation at 13000 rpm for 20 min at 4°C.

**Immunoprecipitation and blotting**

Lysates with adjusted protein concentration (Bradford assay, BioRad) were incubated with antibodies for 2 hours 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 (RK2), rabbit polyclonal anti-RING Cbl/Cbl-b. Immune complexes were precipitated following one-hour incubation with protein A agarose beads. After washing in cold lysis buffer, the complexes were resuspended in Laemmli sample buffer (BioRad), boiled, and resolved by SDS-PAGE.

**Immunofluorescence studies**

Cells were seeded on collagen-coated cover slips for 20 hours, transfected with indicated plasmids using Lipofectamine reagent for 24 hours and starved for additional 12 hours. Cells were left untreated or were stimulated with EGF (100 ng/ml) for 10 min at 37°C and fixed in 4% paraformaldehyde in PBS for 20 min, treated with 50 mM NH₄Cl (10 min) and permeabilized with 0.2% Triton X-100 (10 min). After blocking for one hour in 4% fetal calf serum in PBS, the cells were probed with the primary antibodies (dilution 1:50 or 1:100 in PBS with 2% fetal calf serum, for 90 min) and secondary antibodies conjugated with
fluorophores (60 min). The preparations were mounted using Fluoromount G and the images were taken with Zeiss microscope Axioplan 2.

**Ligand internalization assays**

Ligand internalization assays were performed as described (4) with modifications as described in (1). Briefly, cell monolayers of transfected CHO cells were incubated for 1 hour at 4°C with $^{125}$I-labelled EGF, washed twice with binding buffers and then incubated at 37°C for the indicated time intervals. Cells were transferred on ice and washed with either cold binding buffer or mild acidic buffer to remove surface-bound radiolabelled EGF. The remaining radioactivity in cells was quantified following 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 (wt, wild type) and CIN85 constructs: full size, CIN85-3SH3 or CIN85-PCc 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 every experiment the levels of transfected Cbl-b and CIN85 constructs were analysed in cell lysates.
Results

CIN85 binds to the distal carboxyl termini of Cbl-b and Cbl, but not Cbl-3

We performed a yeast two-hybrid screen searching 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 terminus 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. The result in Figure 1A shows that 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).

In order to further analyze Cbl-b domains that are 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 (Δ927) 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 (Δ891 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 each of these PxxP motifs or all of them together and showed that neither mutation did abrogate 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.

Since 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. While 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 GST binding assays and did not colocalize with CIN85 in transfected NIH-EGFR cells (data not shown). While 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-b 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 (Figure 2B, left panel) as previously described (1,17). The complex between CIN85 and activated receptors was detected as early as 5 minutes and was sustained up to 30 minutes after PDGF stimulation (Fig. 2D). This 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, while 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 downregulation.**

In order to test a functional significance of CIN85 in regulating EGF receptor endocytosis we used a dominant interfering form of CIN85, containing the three SH3 domains (CIN85-SH3) (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 to cells transfected with EGF receptor alone (Fig. 4A). Cbl-b accelerated internalization of
EGF receptors was blocked by expression of CIN85-3SH3, but not CIN85 (Fig. 4A). Comparative assay in CHO cells transfected with either of the three members of Cbl family showed that Cbl and Cbl-b were more potent in inducing EGF receptor internalization than Cbl-3 (Fig. 4B), that is unable to bind to CIN85 (Fig. 1D). This was despite the fact that Cbl-3 was expressed to higher level than the other Cbl proteins (Fig. 4B, right panel). Interfering properties of CIN85 truncation were further demonstrated by immunofluorescence studies, showing that in cells expressing CIN85-3SH3 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 (18)). Similar observations were also reported for CMS, CIN85-related adaptor protein, that 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 downregulation (29).

Since ubiquitination of receptors was implicated as a signal for internalization (4), we checked if 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 that 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 while 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 dermatofibrosarcoma protuberans (DFSP) patients (22), while 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 upregulated by the addition of EGF or PDGF to the medium (Fig. 5A and 5B), 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 (SCF) have been isolated from mast cell tumors and gastrointestinal stromal tumors (GIST). A c-Kit mutation in the tyrosine kinase domain (D816V) is commonly found in human mastocytomas, while a mutation in the juxtamembrane domain (V560G) is associated with GIST (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 Figure 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 SCF. This suggests that CIN85 together with Cbl/Cbl-b are associated with oncogenic RTKs and could thus play an important role in receptor downregulation 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 downregulation of activated receptor tyrosine kinases (Fig. 6). Following ligand binding, RTKs become tyrosine phosphorylated, 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 RTKs endocytosis (1,17). Therefore, Cbl-b appears to promote receptor downregulation 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, while its function is not directly involved in the control of receptor ubiquitination (Fig. 4A, 4C, 4D). Importance of this pathway has been demonstrated in downregulation 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<sup>−/−</sup>-Cbl-b<sup>−/−</sup> mice (unpublished data after (39)), while 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 EGF, PDGF and c-Kit receptors in several tumor cell lines, indicating that this mechanism is also involved in downregulation of oncogenically-activated receptor tyrosine kinases (Fig. 5 and 6). The complex formation seems to be dependent on receptor kinase activity, and all used tumor cell lines 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 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 signal is quickly terminated by removal of receptor molecules from the plasma membrane and subsequent degradation, receptors in malignant cells seem to escape downregulatory 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- all contributing 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 crosslinking 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. Firstly, high affinity binding to Cbl requires multiple SH3 domains of CIN85 (1), and secondly, their association is regulated by tyrosine phosphorylation of Cbl/Cbl-b (Fig. 2) (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. 2B and 2C). 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-containing proteins, and thus to 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 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. Additional interesting feature of both Cbl and CIN85 is that they can oligomerize via their leucine zipper or coiled-coil domains, respectively (18,46). Possibly, 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. As for now, the initial evidence has begun emerging on the redundant role that Cbl/Cbl-b and CIN85 play in regulating multiple steps in downregulation of activated tyrosine kinase receptors.
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References


Figure legends

Fig. 1 **SH3 domains of CIN85 associate with the distal carboxyl terminal part of Cbl-b.**

(A) Yeast clones expressing full size CIN85, 3SH3 or PCc domains were transformed with Cbl-b cDNA (base pairs 2894-3271) and streaked on selective agar plates (-Leu-Trp-His+3-aminotriazole). Growth rate (left) and β-galactosidase activity in a filter lift assay (right) are shown. (B) HA-tagged full size Cbl-b was overexpressed together with FLAG-tagged CIN85-3SH3 or PCc constructs in HEK293T cells. The lysates were immunoprecipitated using anti-FLAG antibody, and the membrane was probed with anti-HA and anti-FLAG antibodies. Expression of transfected proteins was monitored in total cell lysates (TCL). (C) Constructs of Cbl-b: full-size (wt), amino acids 1-927, 1-891, 1-751, 1-349 (N1/3) and 327-982 (C2/3) were transiently expressed together with FLAG-CIN85 in HEK293T cells. Lysates were immunoprecipitated with an Cbl-b (RF) antibody and blotted with anti-RF and FLAG antibodies. (D) CIN85 was transiently expressed together with HA-tagged Cbl, Cbl-b or Cbl-3 in HEK293T cells, and lysates were immunoprecipitated with an anti-HA antibody. The upper panel shows the amount of proteins in the complex, while the lower panel indicates protein levels in total cell lysates. (E) Schematic structure of Cbl-b and CIN85 constructs used in this study. Abbreviations: SH2, unconventional Src homology 2 domain; RF, RING-finger; PxxP, polyproline region; LZ, leucine zipper; DCT, distal carboxyl terminus; SH3, Src homology 3 domains; Cc, coiled coil domain.

Fig. 2. **CIN85 forms inducible complexes with Cbl-b and RTKs.**

(A) HEK293T cells transiently expressing EGFR, HA-Cbl-b and either CIN85 or CIN85-3SH3 were left unstimulated or were stimulated with EGF 100 ng/ml for 10 min. Lysates were immunoprecipitated with an anti-FLAG antibody (M2). Abs, antibody heavy chain (B)
Cells were starved and left untreated or were stimulated with 100 ng/ml EGF for 10 min. Immunoprecipitations using anti-CIN85 antibody (CT) or anti-Cbl-b antibody were performed, and the membranes were probed as indicated, and reprobed after stripping with an anti-phosphotyrosine antibody (PY99). Left panel: HeLa cells; right panel: NIH cells expressing EGF receptor. (C) NIH3T3 cells were stimulated with PDGF (50 ng/ml, 10 min) and lysates were proceeded as in B. (D) NIH3T3 cells were stimulated with PDGF for indicated time points, followed by lysis and immunoprecipitation with an anti-CIN85 antibody. Levels of CIN85 and phosphorylated receptors are shown.

Fig. 3. Relocalization of CIN85 and Cbl-b to internalized complexes with EGF receptors in CHO-EGFR cells upon ligand stimulation. Cells seeded on glass cover slips were starved and treated with EGF for 10 min. Fixed cells were immunostained with anti-EGFR together with anti-Cbl-b or anti-CIN85 (477), and corresponding secondary antibodies conjugated to FITC and TRITC, respectively. Selected areas of co-localization are pointed with arrows. Scale bars correspond to 10 μm.

Fig. 4. A role of CIN85 in Cbl-b-mediated receptor internalization and ubiquitination. (A) Ligand internalization assays were performed as described in Materials and Methods. EGF internalization in CHO cells shows effect of CIN85 and CIN85-3SH3 on Cbl-b-facilitated EGFR internalization. (B) Ligand internalization assay in CHO cells comparing effect of overexpressed Cbl, Cbl-b and Cbl-3 on EGFR internalization. The panel on the right shows levels of Cbl expression in total cell lysate from control (-) and transfected cells (C) CHO cells with stable expression of EGFR were transiently transfected with FLAG-tagged CIN85-3SH3 or CIN85 as indicated. The cells were left unstimulated or were stimulated with 100 ng/ml EGF for 10 min. Immunofluorescence studies on cells expressing FLAG-tagged
constructs were performed with antibodies against FLAG (M2) and EGFR (RK2), the merged picture is shown in the bottom panel. An asterisk points out a characteristic large vesicle formed in cells overexpressing CIN85. (D) HEK293T cells were transiently transfected with EGF receptor, FLAG-ubiquitin, HA-tagged Cbl-b constructs and FLAG-tagged CIN85 constructs; the cells were untreated (-) or stimulated with EGF, 10 min (+) and EGF receptors were immunoprecipitated from the lysates. The top panel shows level of receptor ubiquitination (anti-FLAG) and the same membrane probed with an anti-EGF receptor antibody (RK2), while the bottom panel shows levels of transfected CIN85 and Cbl-b constructs.

Fig. 5. **Receptor complex formation in oncogenically transformed cells.**

(A) DFSP cells were starved and untreated (-) or stimulated with 50 ng/ml PDGF for 10 min (+). Lysates were immunoprecipitated using anti-CIN85 or anti-Cbl-b antibodies and protein complexes were visualized by immunoblotting. (B) A431 cells were untreated (-) or stimulated with 100 ng/ml EGF for 10 min (+). Lysates were immunoprecipitated using an anti-CIN85 antibody. (C) HMC-1.1 and HMC-1.2 cells were untreated (-) or stimulated with 100 ng/ml SCF for 10 min (+). Lysates were immunoprecipitated using anti-CIN85 or anti-Cbl-b (RF) and the protein complexes were visualized by immunoblotting with anti-c-Kit, anti-Cbl-b and anti-CIN85 antibodies.

Fig. 6. **Schematic model of RTKs downregulation in normal and transformed cells.**

Down-regulation of tyrosine kinase receptors is a critical step in modulating their biological activity. This is primarily due to the cessation of receptor-induced signal transduction following Cbl/Cbl-b regulated receptor internalization and degradation. Growth factor binding to its receptor induces receptor autophosphorylation, followed by association of
Cbl/Cbl-b to specific phosphorylated tyrosine residues. In this complex, Cbl proteins are tyrosine phosphorylated, which increases their ability to ubiquitinate active receptors, and enhances their association with adaptor protein CIN85 that in turn regulates internalization of the receptor complex via clathrin-coated pathway. In transformed cells, receptors are constitutively activated and thus complexed with Cbl and CIN85 in absence of external ligand (right). The pathways responsible for such transformation include: (A) autocrine secretion of ligands – i.e. DFSP, (B) receptor dimerization due to high overexpression of receptor at the plasma membrane – i.e. A431, (C) gain-of-function mutations – i.e. HMC-1.1 and HMC-1.2. The receptor/Cbl/CIN85 complex is found in endocytic vesicles, and leads to at least partial receptor downregulation in transformed cells.
Fig. 1.

A

B

C

D

E

Cbl-b

SH2

RF

PxxP

DCT

LZ

Δ927

Δ891

Δ751

N1/2

C2/3

CIN85

SH3

SH3

SH3

PxxP

Cc

CIN85-3SH3

CIN85-PCc
**Fig. 2.**

A

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Anti-HA

Anti-FLAG

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Anti-pTyr1009 PDGFR

Anti-Cbl-b

Anti-CIN85

IP: CIN85
Fig. 3.

control

EGF 10'

 EGFR  Cbl-b  EGFR  CIN85

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Fig. 5.

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Fig. 6.

Normal cells

Transformed cells
CIN85 participates in Cbl-b-mediated downregulation of receptor tyrosine kinases
Iwona Szymkiewicz, Katarzyna Kowanetz, Philippe Soubeyran, Ana Dinarina, Stanley Lipkowitz and Ivan Dikic

J. Biol. Chem. published online August 12, 2002

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