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Negative regulation of EphA2 receptor by Cbl

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Abstract

The *c-Cbl* proto-oncogene product Cbl has emerged as a negative regulator of receptor and non-receptor tyrosine kinases, a function dependent on its recently identified ubiquitin ligase activity. Here, we report that EphA2, a member of Eph receptor tyrosine kinases is negatively regulated by Cbl. The negative regulation of EphA2 mediated by Cbl is dependent on the activity of EphA2, as the kinase inactive mutant of EphA2 cannot be regulated by Cbl. Moreover, a point mutation (G306E-Cbl) in TKB region of Cbl that has been reported to abolish Cbl binding to RTKs and non-receptor tyrosine kinases impaired the binding to active EphA2. The dominant negative mutant 70Z-Cbl, which has a 17-amino acids deletion in the N-boundary of the RING finger domain, defuncted negative regulatory function of Cbl to EphA2. These results demonstrate that the TKB domain and RING finger domain of Cbl are essential for this negative regulation.

Keywords: Eph; Ephrin; Cbl; Phosphorylation; Tyrosine kinase

The Eph receptors constitute the largest subfamily of receptor tyrosine kinases RTKs, with 14 related members. The ligands for Eph RTKs are a family of cell surface-anchored proteins, the ephrins, which are attached to the plasma membrane either via a glycosylphosphatidylinositol (GPI) linkage (class A ephrins) or transmembrane sequence (class B ephrins) [1]. Ephrins are relatively unique in that they need to be membrane-bound to activate Eph receptors, whereas as soluble forms of class A and B ephrins are inactive at Eph phosphorylation. Thus Eph receptors appear to transduce signals initiated by direct cell–cell interactions [2]. A further characteristic of the Eph/ephrin signaling is that the signaling is bidirectional, when Eph receptors bind their cognate ligands ephrin-B or ephrin-A, both Eph and ephrin can transduce signals into intracellular components of cells on which they are displayed [3]. Recent evidence indicates that members of the Eph and ephrin families play critical roles in development of the nervous system, patterning of the embryo and angiogenesis [4–7]. Overexpression of Eph receptors and elevated Eph activity have been found in numbers of tumors, suggesting their role in tumorigenesis [8,9]. However, the mechanisms that regulate activated Eph receptors and their intracellular fate have not been elucidated.

Cbl is a 120 kDa protein product of the proto-oncogene of *c-Cbl*. Recent studies have identified Cbl as a negative regulator of RTKs, such as the epidermal growth factor receptor (EGFR), platelet-derived growth receptor (PDGFR), CSF-1 receptor and c-Met, as well as non-receptor tyrosine kinases such as ZAP-70, Syk, and src-family kinases [10–15]. The N-terminal tyrosine kinase binding (TKB) domain, the RING finger domain and the linker region between the TKB and RING finger domains have been shown to be critical for Cbl-mediated negative regulation [12]. The C-terminal half of Cbl has a proline rich region and several tyrosine residues that are phosphorylated upon receptor stimulation, providing

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potential binding sites for SH3 and SH2 domain-containing proteins. Finally, a leucine zipper domain mediates the formation of Cbl homodimers [13–15]. Recent studies have shown that Cbl functions as a RING-type ubiquitin ligase (E3) towards some of the receptor tyrosine kinases as well as non-receptor tyrosine kinases [16–18], but information is still sparse as to the varieties of kinases. Given the diversity of the Eph subfamily of receptor tyrosine kinase and relative lack of knowledge about mechanism of their regulation, we examined whether Cbl regulates the activity of EphA2 and other members of this family. Our results show that EphA2, but not EphB1 or EphB2, is negatively regulated by Cbl. As to negative regulation of EphA2, it seems to be similar as in case with EGFR, considering the various effects of several Cbl constructs. However, the presumable target portion of Cbl in the receptors has not been determined yet, the uniqueness of our observation is still unknown. Furthermore, we do not know what kind of differences between EphA2 and EphB1/2 makes the different regulation by Cbl. Testing several recombinant receptors are in progress.

Materials and methods

Construction of plasmid and preparation of fusion protein. The details of the constructions are in supplementary information 1. The pAlterMax-based Fc fusion protein expression constructs of ephrin-B1 and ephrin-A1 (Fig. 1A) were derived by triple ligation of sequences encoding the ephrin extracellular domains (as *Eco*RI–*Bam*HI fragments) and Fc fragment of mouse IgG2b (as a *Bam*HI–*Xba*I fragment) into pAlterMax. The methods used here were described previously [19]. The sequences of extracellular domain of ephrin-A1 was amplified from a cDNA template derived from 293T cells, which express low levels of ephrin-A1 protein. Fc fragment of mouse IgG2b was amplified from pcDNA–ephrin-B1–Fc (a gift from Dr. Ikegaki). Preparation and stimulation are described in the supplementary information 2. The concentrations of fusion proteins were measured with a BioRad Protein Assay Kit (BioRad laboratories, CA), and their integrity and purity were established by Coomassie blue staining after resolution on SDS–PAGE gels (Fig. 1B). The ability of ephrin–Fc fusion proteins was assessed by induction of tyrosine phosphorylation of EphA2 and EphB2, respectively.

Antibodies. Polyclonal anti-Cbl (C-15), anti-EphB2 (C-20), anti-EphB1 (M-19), and anti-HA (Y-11) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-EphA2 and anti-phosphotyrosine (4G10) were from Upstate Biotechnology (Upstate, NY).

Eph receptor stimulation. Stimulation procedures are in the supplementary information 3.

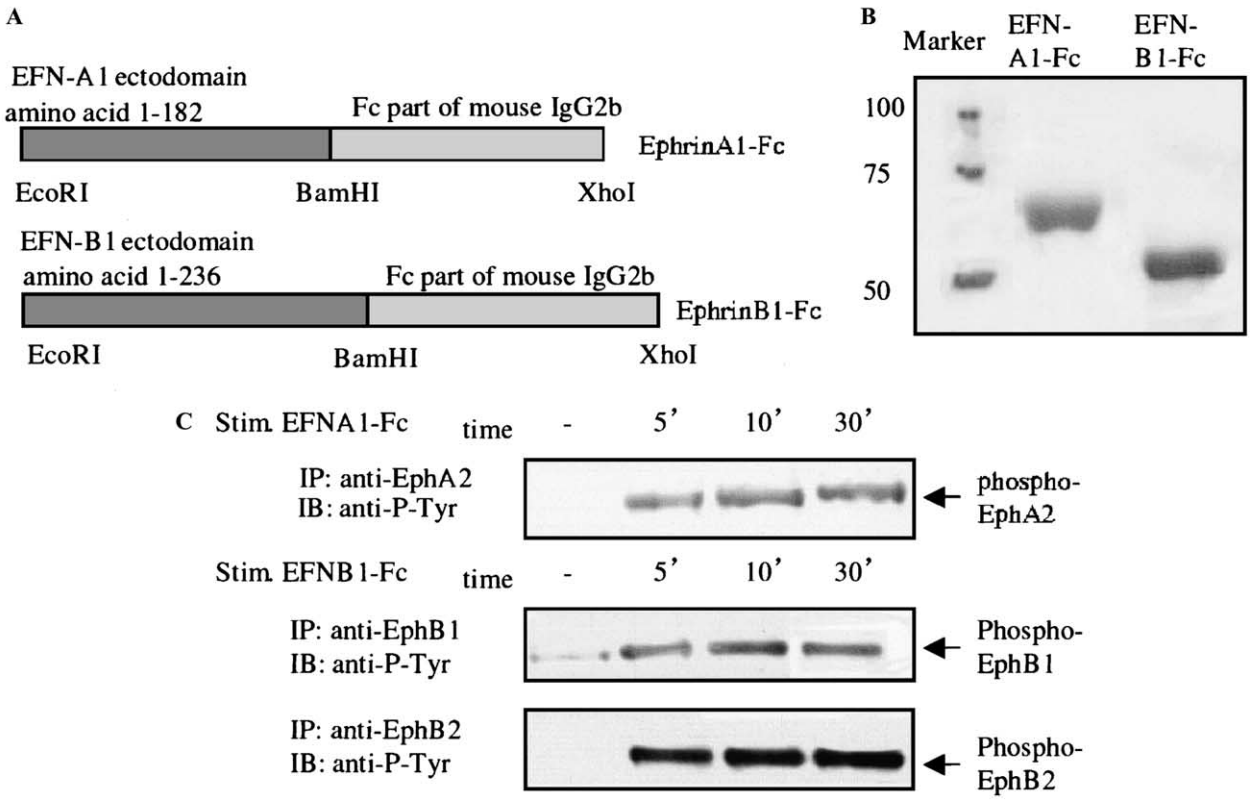

Fig. 1. Characterization of ephrin–Fc fusion proteins used as ligands for Eph stimulation. (A) Schematic representation of ephrin–Fc fusion proteins (see Materials and methods for details). The numbers refer to residues of ephrin-A1 and ephrin-B1 used for fusion to mouse IgG2b Fc. (B) EFNB1–Fc and EFNA1–Fc fusion proteins isolated from transfected 293T cells. Fusion proteins were purified for supernatants of transfected cells as described in Materials and methods. One μg aliquots were analyzed by SDS–polyacrylamide gel electrophoresis followed by Coomassie blue staining. Polyacrylamide gel electrophoresis of supernatant of the cells transfected with EFNB1–Fc and EFNA1–Fc (Coomassie blue stain). (C) Biological activity of ephrin–Fc fusion protein. 293T cells transfected with EphA2, EphB1, or EphB2 as indicated were stimulated with EFNA1–Fc or EFNB1–Fc for the indicated time. Aliquots of lysate protein were immunoprecipitated with appropriate anti-receptor antibodies and immunoblotted with anti-phosphotyrosine.

Cell culture and transfection. Human embryonic kidney (HEK) 293T cells were cultured as described previously [20]. Cells (293T) stably expressing EphB1 or EphB2 were cultured in DMEM containing 10% fetal bovine serum and 2 μg/ml puromycin (Sigma, Germany). Transfection was performed using the calcium phosphate coprecipitation method with concurrent treatment with 25 μM chloroquine, as described [20]. Culture medium was replaced at 8 h following the addition of DNA precipitates. Cells were harvested at 48 h following the addition of DNA precipitates. The amount of the input DNA in each transfection was equalized with empty vector DNA.

Western blotting and immunoprecipitation. Western blotting and immunoprecipitation were performed in an ordinary way (supplementary information 4).

In vitro binding assay. In vitro binding assay was performed according to the protocol published previously [21] and the supplementary information 5.

Results

Negative regulatory effect of Cbl on EphA2, but not on EphB1 or EphB2

Under the specific transfection conditions used, substantial activation of overexpressed EphA2, EphB1, and EphB2 receptors was observed, as demonstrated by its autophosphorylation in anti-phosphotyrosine blots (Fig. 1C). Importantly, overexpression of increasing amounts of Cbl resulted in a Cbl dose-dependent decrease in the level of EphA2 phosphorylation signal, concomitantly, a reduction in the level of EphA2 protein was observed (Fig. 2A). Notably, the effect of Cbl was selectively observed with EphA2, as co-expression of Cbl did not decrease the level of protein or phosphorylation of EphB1 or EphB2 (Figs. 2B and C). As the overall level of autophosphorylation of EphB1 and EphB2 in these experiments was lower than that of EphA2, we wished to exclude the possibility that lack of an effect of Cbl on these receptors might be due to their lower level of phosphorylation. For this purpose, 293T cells transformed with EphB1 or EphB2 were transiently transfected with vector or Cbl expression construct, and analyzed with or without stimulation with clustered ephrin-B1–Fc. As shown in Fig. 2D in supplementary information, overexpression of Cbl had no effect on the level of phosphorylated EphB1 and EphB2 or their protein levels. These findings further substantiate our conclusion that the effect of Cbl on EphA2 is selective. Given the observation above that Cbl expression induced the loss of autophosphorylated EphA2, and recent observation of the kinase activity of EGFR is required for its susceptibility to Cbl, we tested whether the Cbl-mediated effect on EphA2 requires the activity of EphA2. For this purpose, the EphA2 kinase-inactive mutant EphA2-K646M was co-expressed with Cbl under conditions comparable to those used in Fig. 2A. As anticipated, the kinase inactive EphA2 showed no detectable autophosphorylation, whereas autophosphorylation of wild-type EphA2 was readily detectable. Notably, co-expression of increasing amounts of Cbl did not reduce the level of the EphA2-K646M mutant protein (Fig. 2E, in the supplementary information). Thus, the observed effect of Cbl to induce the loss of EphA2 protein is dependent on EphA2 kinase activity.

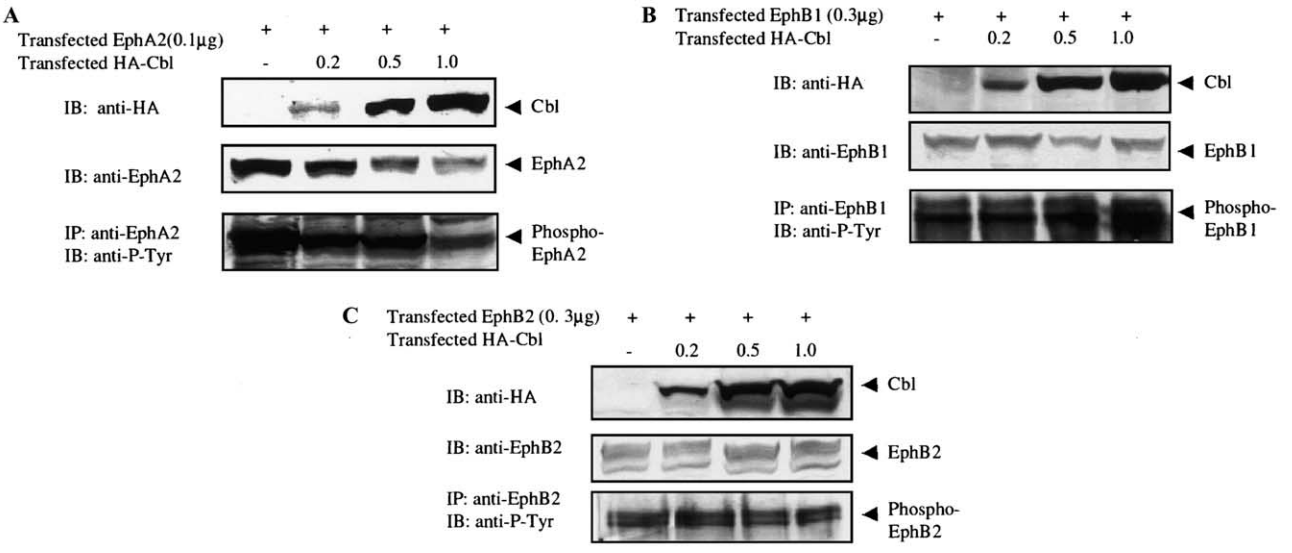

Fig. 2. Cbl-mediated negative regulation of EphA2, but not EphB1 and EphB2. Cells (293T) on 60 mm plates were transiently transfected (A–C) with 0.1 μg of vectors encoding EphA2 (A) and 0.3 μg of EphB1 or B2 (B, C) together with the indicated amounts of a vector encoding HA-Cbl. Cell lysates (50 μg/lane) were separated by SDS–PAGE and analyzed by Western blotting with indicated first antibodies (upper and middle panel). Alternately, aliquots were subjected to immunoprecipitation with the indicated anti-Eph receptor antibodies followed by anti-phosphotyrosine (anti-P-Tyr) immunoblotting. The expression of transfected Cbl was detected by immunoblotting with anti-HA antibody.

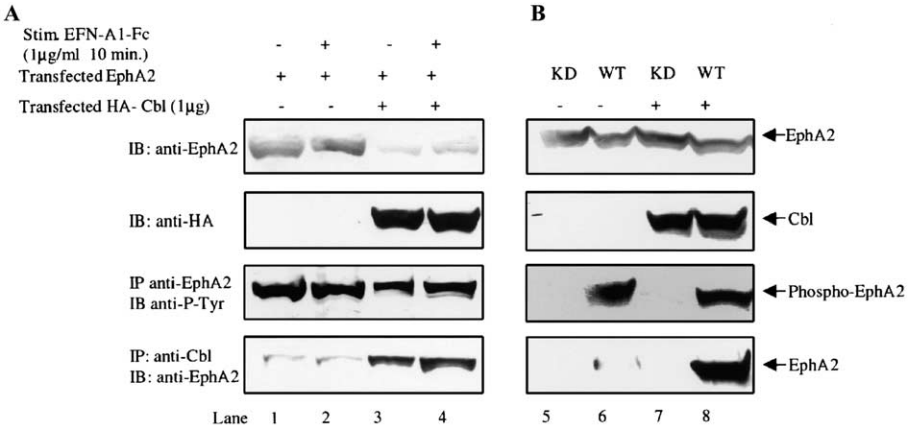

Fig. 3. Association of EphA2 and Cbl and dependence of association on the kinase activity of EphA2. (A) Cells (293T) were transiently cotransfected with vectors encoding EphA2 and HA-Cbl, 48 h after transfection, the cells were serum starved and stimulated with clustered EFN-A1–Fc (1 μg/ml) for 10 min. Cell lystes (50 μg/lane) were separated by SDS–PAGE and immunoblotted with the indicated antibodies to examine the expression of tansfected proteins. Aliquots of lysates protein were immnoprecipitated with an anti-Cbl antibody and subjected to immunoblotting with an anti-EphA antibody to assess association with EphA2. (B) As in (A), except that the wild-type (WT) or kinase-dead (KD) EphA2 constructs were used as indicated.

Association of Cbl with EphA2 and its dependence on EphA2 phosphorylation

To assess the potential Cbl–EphA2 association in vivo, EphA2 and Cbl were transiently cotransfected into 293T cells, and lysates of unstimulated or ephrin-A1–Fc-stimulated cells were subjected to anti-Cbl immunoprecipitations followed by anti-EphA2 immunoblotting. As shown in Fig. 3A, lanes 1 and 2, a low, but detectable EphA2 signal was observed in anti-Cbl immunoprecipitates of 293T cells where exogenous Cbl was not introduced reflecting EphA2 association with endogenous Cbl. Notably, a markedly higher level of EphA2 was co-immunoprecipitated with Cbl in cells when exogenous Cbl was introduced even though a vastly decreased level of EphA2 is present in these cells (Fig. 3, lane 3 and 4). Even though a substantial level of EphA2–Cbl interaction was observed in the absence of ligand stimulation, given the increased level of autophosphorylation of EphA2, a noticeable increase in association was observed upon ephrin-A1–Fc stimulation (e.g. compare lanes 3 and 4 in Fig. 3A), suggesting that activation of EphA2 promoted its interaction with Cbl. Consistent with this interpretation, no interaction was observed between kinase-inactive EphA2 and Cbl (Fig. 3B). Finally, we did not observe an association of Cbl with EphB1 or EphB2, regardless of whether the wild-type or kinase inactive form of these two receptors were used (data not shown). Thus, the association of Cbl with Eph receptors strongly correlates with its ability to negatively regulate them, and is dependent on the receptor kinase activity.

The TKB domain of Cbl is essential for the negative regulation of EphA2

Previous studies have indicated that N-terminal tyrosine kinase-binding (TKB) domain plays a critical role in the association of Cbl with activated RTKs as well as in their negative regulation. Indeed, a Cbl mutant Cbl-G306E carrying an inactivating point mutation in the TKB domain fails to negatively regulate RTKs such as PDGFR and EGFR [12]. We wished to examine the role of the Cbl-TKB domain in Cbl-mediated EphA2 regulation. When Cbl-G306E (TKB domain mutant) was co-expressed with EphA2, no loss of EphA2 protein was observed nor was a diminution of its phosphorylation (Fig. 4A, top panel and third panel). Importantly, the co-immunoprecipitation analysis showed that Cbl-G306E failed to interact with EphA2 (Fig. 4A, bottom panel).

To directly test whether the TKB domain itself associates with EphA2, we co-expressed EphA2 with HA-tagged Cbl-TKB domain (residues 1–357) either in their wild-type construct (Cbl-N) or carrying the inactivating mutation G306E (Cbl-N-G306E). While EphA2 was easily co-immunoprecipitated with HA-Cbl-N, essentially no association was detected with HA-Cbl-N-G306E, even though both proteins were expressed at comparable levels (Fig. 4B). To further establish the ability of the Cbl-TKB domain to bind to EphA2, in vitro binding assays using Cbl-TKB domain-containing GST fusion proteins were carried out. For this purpose, GST, GST–Cbl-1-436, and GST–Cbl-1-436-G306E were purified from bacteria (Fig. 4C) and non-covalent coated on glutathione–Sepharose beads, and used to pull down the EphA2 receptor from lysate of 293T cell transfected with EphA2. While the GST control showed no binding to EphA2, as expected, GST–Cbl-1-436 prominently bound to EphA2; this binding was markedly reduced by the G306E mutation (Fig. 4D). Although the GST fusion protein used here contained the RING finger domain, given the Cbl-N (WT) (Fig. 5A) and RING finger mutant 70Z-Cbl to associate with

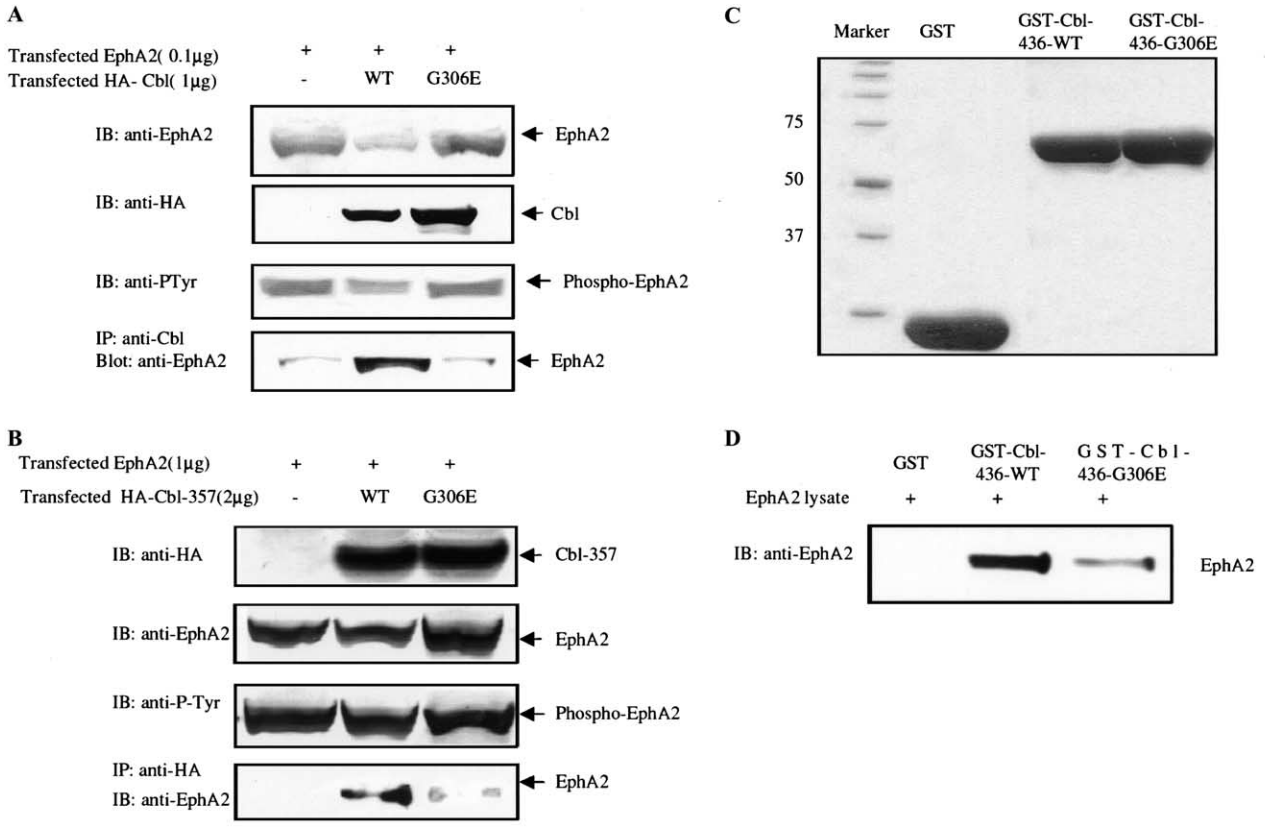

Fig. 4. The role of Cbl-TKB domain in the negative regulation of EphA2 and in Cbl–EphA2 association. (A) Cells (293T) were transiently transfected with vectors encoding EphA2 and HA-Cbl-G306E as indicated. After 48 h, cells were lysed, the transfected proteins were detected by immunoblotting with the indicated antibodies. Aliquots of lysate protein were subjected to immunoprecipitation with an anti-Cbl antibody and the presence of EphA2 in immunoprecipitates was detected with an anti-EphA2 antibody (bottom panel). (B) Cells (293T) transiently transfected with vectors encoding EphA2 together in HA-Cbl-1-357 (Cbl-N) HA-Cbl-1-357-G306E (Cbl-N-G306E). After 48 h, cells were lysed and expression of transfected protein was detected by immunoblotting with the indicated antibodies. Aliquots of lysate protein were subjected to immunoprecipitation with an anti-HA antibody and the presence of EphA2 in immunoprecipitates was detected with an anti-EphA2 antibody. (C) In vitro binding of EphA2 to GST–fusion protein containing the Cbl-TKB domain. GST-Cbl-1-436 and GST-Cbl-1-436-G306E proteins were prepared according to the procedures mentioned in Materials and methods. Five-hundred μg aliquots of lysate protein from EphA2 expressing 293T cell transfectants were incubated with 10 μg each of the indicated GST fusion proteins noncovalently adsorbed to glutathione–agarose for about 1 h at 4 °C and washed four times with washing buffer. Bound proteins were resoluted by SDS–PAGE and the presence of EphA2 was detected by Western blotting with an anti-EphA2 antibody.

EphA2 (Fig. 5B), our results demonstrate that the TKB domain of Cbl mediates Cbl–EphA2 association and is essential for Cbl-dependent loss of EphA2 protein.

An intact RING finger domain is essential for Cbl-mediated negative regulation of EphA2

Recent results have established that Cbl-TKB domain-mediated binding to RTKs and certain non-receptor tyrosine kinases (e.g. Syk and ZAP70) allows Cbl to facilitate the ubiquitination of bound tyrosine kinases; an intact RING finger domain, which binds to ubiquitin conjugating enzymes and is required for Cbl's ubiquitin ligase activity, is required for target tyrosine kinase ubiquitination and subsequent degradation. Given our observation that interaction with Cbl promoted the loss of EphA2 protein, we wished to establish if this was Cbl RING finger domain-dependent. For this purpose, we co-expressed EphA2 with 70Z-Cbl which carries a 17-amino acids (amino acids 366–382) deletion within the linker region connecting the TKB and RING finger domains; this deletion is known to abrogate the E3 ubiquitin ligase activity of Cbl and to impair a dominant-negative phenotype on the mutant [22]. When 70Z-Cbl and EphA2 were co-expressed in 293T cells, EphA2 levels did not decrease; in contrast, the levels showed an increase with increase of 70Z-Cbl. Expression of EphA2 and the phosphorylation of EphA2 also increased (Fig. 5A). 70Z-Cbl-induced increase in EphA2 levels was not observed when kinase-inactive EphA2 (K646M) was used (data not shown). As expected from the presence of an intact TKB domain in 70Z-Cbl, this mutant showed an intact association with EphA2 (Fig. 5B). The observation that overexpression of 70Z-Cbl led to an increase in EphA2 levels is consistent with the dominant-negative mode of action of this mutant by

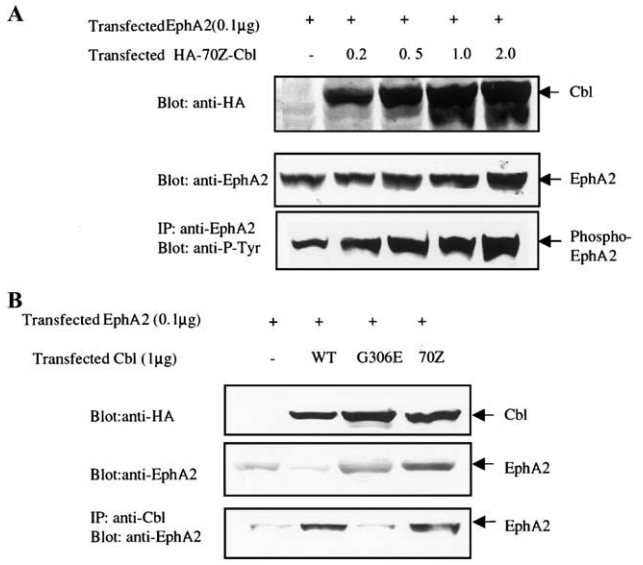

Fig. 5. An intact of Cbl RING finger domain is essential for Cbl-mediated negative regulation of EphA2. (A) Cells (293T) were transiently transfected with vectors encoding HA-70-Cbl and EphA2. After 48 h, cells were lysed and the expressions of transfected protein were detected by immunoblotting of lysates with the indicated antibodies. (B) Cells (293T) were transiently transfected with vectors encoding EphA2, HA-Cbl(-) and HA-Cbl-G306E, or HA-70Z-Cbl. After 48 h, cells were lysed and expression of transfected protein was detected by immunoblotting with indicated antibody. The lysates were subjected to immunoprecipitaion with an anti-Cbl antibody and the presence of EphA2 in immunoprecipitates was detected by immunoblotting with an anti-EphA2 antibody.

preventing EphA2 degradation through ubiquitination by endogenous Cbl.

Discussion

The Eph receptor tyrosine kinases constitute the largest known family of RTKs, and play essential roles in embryogenesis, neurogenesis, angiogenesis, and tumorigenesis [4–7]. However, relatively little is known about how members of this subfamily of RTKs are up- or down-regulated. Recent analyses have identified Cbl oncoprotein as a critical regulator of lysosomal targeting of RTKs such as EGFR. Thus, we wished to assess if Cbl participates as a negative regulator of Eph receptors too. Studies reported here establish that this is indeed the case. Interaction with Cbl appears to be primarily mediated by the Cbl TKB domain binding to autophosphorylated EphA2. Consistent with this mechanism of interaction, a Cbl mutant with an inactivating mutation in the TKB domain failed to associate with EphA2. Furthermore, the Cbl-TKB domain alone was able to co-precipitate with EphA2, and bound to EphA2 also in vitro.

Using a 293T cell co-expression system, we demonstrated that Cbl selectively associate with EphA2, but not with EphB1 or EphB2. In each case, the binding was abolished by G306E mutation. The evolutionarily conserved TKB domain of Cbl is required for binding to other activated tyrosine kinases, in which it binds to specific phosphorylation sites. A similar mechanism is likely for Cbl–EphA2 association as the kinase activity of EphA2 was found to be essential for association with Cbl. The identity of potential Cbl-TKB domain binding sites on EphA2 will require further investigation and should be helpful in dissecting the role of Cbl-mediated regulation in controlling the activity of EphA2, and in determining the basis for selective association of EphA2, as compared to EphB1 and B2, with Cbl.

Our analysis in 293T overexpression system provides strong evidence that Cbl can negatively regulate the activity of EphA2 receptor. Co-expression with wild-type Cbl, but not with non-interacting Cbl-G306E mutant, led to loss of autophosphorylated EphA2. The loss of EphA2 protein, apparently a result of degradation, was specific as it was not observed with EphB1 or B2, nor with the kinase-inactive EphA2, which failed to associate with Cbl. When we co-expressed EphA2 with 70Z-Cbl, not only did we not observe any decrease in the levels of EphA2 protein; in fact a 70Z-Cbl dose-dependent increase in the levels of EphA2 was seen. Such an increase was only seen when active EphA2 was used.

Taken together, Cbl-mediated EphA2 degradation seems to be similar as in case with some of the RTK, but Cbl-mediated negative regulation among Eph family members is selective, tempting us to elucidate how this discrimination occurs. Anyway, this suggests the possibility that the interaction with Cbl may control the outcome of stimulation through different Eph receptors.

Acknowledgments

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