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PTK6 promotes degradation of c-Cbl through PTK6-mediated phosphorylation

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ABSTRACT

PTK6 (also known as Brk) is an intracellular tyrosine kinase which induces proliferation, anti-apoptosis, migration, and anchorage-independent growth. Herein we report that PTK6 phosphorylates and down-regulates E3 ubiquitin ligase c-Cbl. Tyr^{700}, Tyr^{731}, and Tyr^{774} residues in the C-terminal domain of c-Cbl are major phosphorylation sites targeted by PTK6. The phosphorylated c-Cbl is subjected to auto-ubiquitination and degraded through the ubiquitin–proteasome pathway. These results provide evidence for a novel mechanism demonstrating the oncogenic potential of PTK6 through degradation of c-Cbl, which is an E3 ligase important in down-regulation of oncoproteins.

1. Introduction

PTK6, also known as breast tumor kinase (Brk), is a non-receptor type protein tyrosine kinase (PTK) containing an SH3 domain, an SH2 domain, and a tyrosine kinase catalytic domain [1,2]. PTK6 was first identified in melanoma cells [3], and full-length cDNA of human PTK6 was cloned from human breast carcinoma cells [1]. PTK6 is closely related to the Src kinase family, although it seems to be evolutionarily divergent [2]. Increased expression of PTK6 has been observed in various cancer types, including breast, ovarian, colon, head and neck, and prostate cancer [4,5].

It has been reported that PTK6 plays a role in cancer progression by enhancing proliferation, anchorage-independent growth, cell migration, and tumor growth [4,5]. PTK6 is activated by receptor tyrosine kinases (RTKs) that are induced by growth factors such as epidermal growth factor (EGF), heregulin, and hepatocyte growth factor (HGF) [6–8]. Expression of PTK6 makes human mammary epithelial cells more sensitive to EGF-induced mitogenic effects [6] and enhances phosphatidylinositol 3-kinase pathway signaling by increasing phosphorylation of ErbB3 [9]. We have recently shown that PTK6 phosphorylates ARAP1, which decreases endocytosis of EGFR, and thus enhances EGF-induced signaling [10]. PTK6 also enhances HGF/Met receptor signaling by activating Erk5 in breast cancer cells [8]. In addition, the subcellular location of PTK6 is important for its oncogenic potential because PTK6 targeted to the plasma membrane showed strong oncogenic abilities whereas PTK6 targeted to the nucleus did not [11].

c-Cbl is an E3 ubiquitin ligase containing a tyrosine kinase binding (TKB) domain, a RING finger domain, a proline-rich (PR) domain, and a ubiquitin-associated domain [12]. Thus, c-Cbl plays a role in ubiquitination and negative regulation of its target proteins. Target proteins of c-Cbl include RTKs such as the EGF and platelet-derived growth factor (PDGF) receptors [13,14], non-receptor PTKs such as Syk, Fyn, and Lyn, and various signaling proteins such as Vav, STAT5, Notch, and Bim [12]. Further, Cbl itself can be a target for proteasomal degradation, through ubiquitination by its own ligase or other E3 ubiquitin ligases. It has been reported that Src phosphorylates c-Cbl and promotes degradation of c-Cbl by enhancing auto-ubiquitination [15]. In addition, Itch and NEDD4, which are WW domain HECT E3 ubiquitin ligases, ubiquitinylate Cbl for proteasomal degradation [16].

We hypothesized that PTK6, which is closely related to Src, may increase oncogenic potential through down-regulation of c-Cbl. Thus, we analyzed whether c-Cbl is a substrate of PTK6 and whether PTK6 influences the levels of c-Cbl. After this, we determined which tyrosine residues within the c-Cbl domain are phosphorylated by PTK6. Phosphorylation-defective and E3 ligase-defective c-Cbl mutants were generated and analyzed for PTK6-mediated down-regulation. This allowed us to deduce a novel molecular mechanism for the PTK6-mediated enhancement of oncogenic potential through phosphorylation and down-regulation of c-Cbl.

Abbreviations: Brk, breast tumor kinase; EGF, epidermal growth factor; HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor; PR, proline-rich; PTK, protein tyrosine kinase; RTK, receptor tyrosine kinase; TKB, tyrosine kinase binding.

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2. Materials and methods

2.1. Antibodies and reagents

Anti-PTK6 (Brk), anti-Cbl, anti-β-actin, and anti-HA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology (Billerica, MA, USA). Anti-GAPDH antibody was purchased from AB Frontier (Seoul, Korea). Anti-penta-His antibody and Ni^{2+}-NTA agarose were purchased from QIAGEN (Valencia, CA, USA).

2.2. Expression constructs

Human PTK6 expression vectors pcDNA3.1-PTK6, pcDNA3.1-Flag-PTK6, and pcDNA3.1-Flag-PTK6-K219M (kinase-dead mutant) have been described previously [11,17,18]. Expression constructs for variants of His-tagged c-Cbl were generated in pcDNA4-HisMax-c-Cbl using primer pairs (listed in Supplementary Table 1) by site-directed mutagenesis with a QuickChange® kit (Stratagene, La Jolla, CA, USA). His-tagged c-Cbl, HA-tagged c-Cbl, and HA-tagged c-Cbl-C381A expression vectors were generously provided by Prof. Chin Ha Chung (Seoul National University, Korea). All constructs were confirmed by DNA sequencing.

2.3. Cell culture and transfection

HEK293 cells, HEK293T cells, and human breast carcinoma BT-474 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For transient expression, HEK293 cells or HEK293 cells stably expressing PTK6 were transfected with each of the indicated expression constructs using Lipofectamine (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendations. To achieve stable expression, HEK293 cells were transfected with each expression construct using the calcium phosphate method, followed by selection with 1200 μg/ml of G418. After 2 weeks, G418-resistant colonies were cloned and expanded.

2.4. Pull-down assays and Western blot analysis

For EGF stimulation, subconfluent cells were starved in a serum-free medium for 24 h and then stimulated with 50 ng/ml EGF for 10 min. The cells were washed twice with ice-cold phosphate-buffered saline and lysed on ice for 10 min in lysis buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 0.05% protease inhibitor cocktail (Cat. No. P8340, Sigma)] and centrifuged at 10,000×*g* for 10 min at 4 °C. Immunoprecipitation, pull-down assay, and Western blot analysis were performed as described previously [10,18].

2.5. In vitro kinase assay

A constitutively active PTK6 mutant fused to GST (GST-PTK6-Y447F) was purified from *Escherichia coli* harboring pGEX-4T3-PTK6-Y447F as described previously [19]. His-c-Cbl, as expressed in HEK293 cells, was enriched by pull-down using Ni^{2+}-NTA agarose. *In vitro* kinase assay was performed by incubation of purified GST-PTK6-Y447 (160 ng) and His-c-Cbl (200 ng) bound to Ni^{2+}-NTA agarose with 500 μM adenosine-5′-triphosphate (ATP) in kinase reaction buffer (20 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 50 μM Na_3VO_4) for 30 min at 30 °C. The reactions were terminated by adding sodium dodecyl sulfate sample buffer and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot.

2.6. In vivo ubiquitination assay

HEK293 cells, stably expressing Flag-PTK6 or transfected with empty vector, were transfected with HA-ubiquitin and/or His-c-Cbl expression vectors, followed by treatment with 20 μM MG132 for 1 h. His-c-Cbl was pulled down from the cell lysate with Ni^{2+}-NTA agarose, followed by Western blotting with the HA antibody to detect the ubiquitinated c-Cbl.

2.7. Knockdown of PTK6

To silence expression of PTK6, pLKO.1-PTK6-shRNA 1064 (TRCN000021552), pLKO.1-PTK6-shRNA 1866 (TRCN000021549), or pLKO.1-control plasmid (Sigma–Aldrich, St. Louis, MO, USA) was co-transfected in HEK293T cells, together with packaging vector psPAX2 (Addgene, Cambridge, MA, USA) and envelope vector pMD2.G (Addgene). BT-474 cells were infected with PTK6 knockdown or control lentivirus, according to the manufacturer's recommendations (Addgene).

3. Results

3.1. PTK6 phosphorylates c-Cbl and induces down-regulation of c-Cbl

To examine whether c-Cbl is phosphorylated by PTK6, HEK293 cells stably expressing PTK6 or transfected with empty vector were stimulated with EGF. Phosphorylation of endogenous c-Cbl was analyzed using immunoprecipitation and Western blot (Fig. 1A). Upon EGF stimulation, phosphorylation of c-Cbl was drastically increased in PTK6-expressing cells, compared to the empty vector controls. To analyze whether PTK6 also phosphorylates c-Cbl in normal culture conditions, His-c-Cbl was transiently transfected into HEK293 cells stably expressing either PTK6 or empty vector, and verified using pull-down assay. Phosphorylation of His-c-Cbl was dependent on the overexpression of PTK6 (Fig. 1B). Interestingly, we also observed that c-Cbl was down-regulated when it was phosphorylated by PTK6 (Fig. 1A and B).

3.2. Catalytic activity of PTK6 is required for down-regulation of c-Cbl

To examine whether catalytic activity of PTK6 is essential for c-Cbl down-regulation, wild-type Flag-PTK6, or Flag-PTK6-K219M, which is a catalytically inactive mutant, was expressed in HEK293 cells. The level of c-Cbl gradually decreased with increasing levels of wild-type PTK6, but remained unaffected by the expression of kinase-dead PTK6-K219M (Fig. 2A). When His-c-Cbl was co-expressed with Flag-PTK6 or Flag-PTK6-K219M in HEK293 cells, His-c-Cbl was phosphorylated by wild-type PTK6 and down-regulated. As hypothesized, the kinase-dead PTK6-K219M mutant did not show phosphorylation or down-regulation of His-c-Cbl (Fig. 2B). In addition, *in vitro* kinase assay showed that PTK6 directly phosphorylates c-Cbl (Fig. 2C). Together, these results indicate that catalytically active PTK6 phosphorylates, and thereby degrades c-Cbl.

3.3. PTK6 knockdown increases levels of c-Cbl in breast carcinoma cells

The effect of PTK6 on the levels of c-Cbl was analyzed in human breast carcinoma cell line BT-474, which endogenously expresses PTK6 and c-Cbl. When expression of endogenous PTK6 was silenced using a PTK6 shRNA lentivirus, the levels of c-Cbl increased (Supplementary Fig. 1). From this we confirm that PTK6 also induces degradation of c-Cbl in breast carcinoma cells, consistent with the results observed in HEK293 cells that overexpress PTK6.

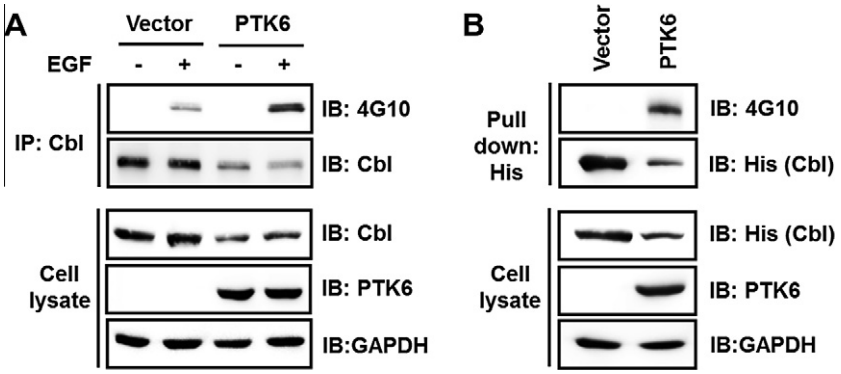

Fig. 1. Analysis of phosphorylation of c-Cbl by PTK6. (A) HEK293 cells stably expressing wild-type PTK6 or empty vector were starved in serum-free medium for 24 h and stimulated with 50 ng/ml EGF for 10 min. Endogenous c-Cbl was subjected to immunoprecipitation (IP) with anti-Cbl antibody. (B) His-c-Cbl was transiently expressed in HEK293 cells stably expressing either wild-type PTK6 or empty vector. After culturing under normal conditions, His-c-Cbl was pulled down using Ni^{2+}-NTA agarose. Phosphorylation of endogenous c-Cbl and His-c-Cbl were analyzed by Western blotting using anti-phosphotyrosine antibody. GAPDH was used as protein loading control.

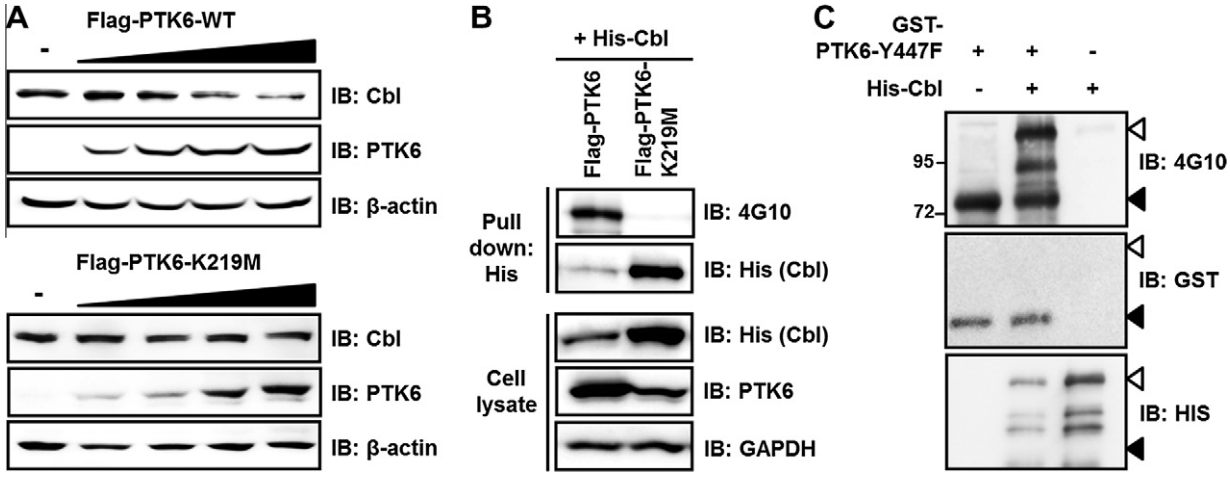

Fig. 2. Effect of PTK6 catalytic activity on the c-Cbl down-regulation. (A) HEK293 cells were transiently transfected with increasing amounts (0, 0.5, 1, 2, and 4 μg per 60-mm dish) of wild-type Flag-PTK6, or kinase-dead Flag-PTK6-K219M expression vector. Expression levels of Flag-PTK6, Flag-PTK6-K219M, or endogenous c-Cbl were analyzed by Western blotting using anti-PTK6 and anti-Cbl antibodies. (B) His-c-Cbl was co-expressed with Flag-PTK6 or Flag-PTK6-K219M in HEK293 cells and pulled down with Ni^{2+}-NTA agarose. Phosphorylation of c-Cbl by PTK6 was detected by Western blotting using an anti-phosphotyrosine antibody. (C) Phosphorylation of c-Cbl by PTK6 was analyzed using an *in vitro* kinase assay. His-c-Cbl, pulled down with Ni^{2+}-NTA agarose, was incubated with or without purified constitutively active PTK6 mutant protein (GST-PTK6-Y447F) in the presence of ATP in kinase reaction buffer. The reaction products were analyzed by Western blotting using anti-phosphotyrosine, anti-GST, and anti-penta-His antibodies. Open and closed arrow heads indicate positions for His-c-Cbl and GST-PTK6-Y447F, respectively.

3.4. C-terminal tyrosine residues of c-Cbl are important for PTK6-mediated phosphorylation and down-regulation of c-Cbl

To identify the domains of c-Cbl that are involved in the observed PTK6-mediated phosphorylation and down-regulation, various His-c-Cbl constructs were expressed in HEK293 cells stably expressing either PTK6 or empty vector (Fig. 3A). Wild-type His-c-Cbl was attenuated by PTK6 expression, whereas the levels of His-c-Cbl-TKB, His-c-Cbl-TKB-RING, and His-c-Cbl-TKB-RING-PR were not affected (Fig. 3B). This indicates that the C-terminal region of c-Cbl is essential for the PTK6-induced down-regulation of c-Cbl.

Web-based prediction tools NetPhos 2.0 [20] and KinasePhos [21] were used to identify potential phosphorylation sites on the C-terminal region of c-Cbl, yielding tyrosine residues Tyr^{700}, Tyr^{731}, and Tyr^{774}. Notably, these sites have been reported to be major phosphorylation sites by PTKs including Src, Fyn, and Syk [22,23]. Consequently, we examined which of the identified c-Cbl tyrosine residues is important for its down-regulation, using single or triple mutants of Y700F, Y731F, and Y774F. His-c-Cbl levels were increased slightly by expression of Y700F and Y731F, and drastically by expression of Y774F (Fig. 3C). Therefore, although Tyr^{774} is the most important site for phosphorylation and down-regulation of c-Cbl, Tyr^{700} and Tyr^{731} are also involved. In addition, expression of a triple mutant of Y700F/Y731F/Y774F (3YF) in HEK293 cells expressing PTK6 nearly abolished the PTK6-induced phosphorylation and degradation of c-Cbl (Fig. 3D).

3.5. Phosphorylation of c-Cbl by PTK6 enhances its proteasome-dependent degradation

We next examined whether the PTK6-induced down-regulation of c-Cbl is mediated by the ubiquitin–proteasome pathway. Wild-type or a 3YF-mutant of His-c-Cbl was co-expressed with HA-ubiquitin in HEK293 cells that stably express PTK6 or empty vector, and the His-c-Cbl levels were analyzed after treatment of MG132, a proteasome inhibitor. We found that PTK6 significantly enhanced the polyubiquitination of wild-type c-Cbl but not that of the 3YF-mutant (Fig. 4A), suggesting that degradation of c-Cbl is indeed achieved through the ubiquitin–proteasome pathway.

Furthermore, we know that c-Cbl contains a RING finger domain, which has E3 ubiquitin ligase activity. To examine whether

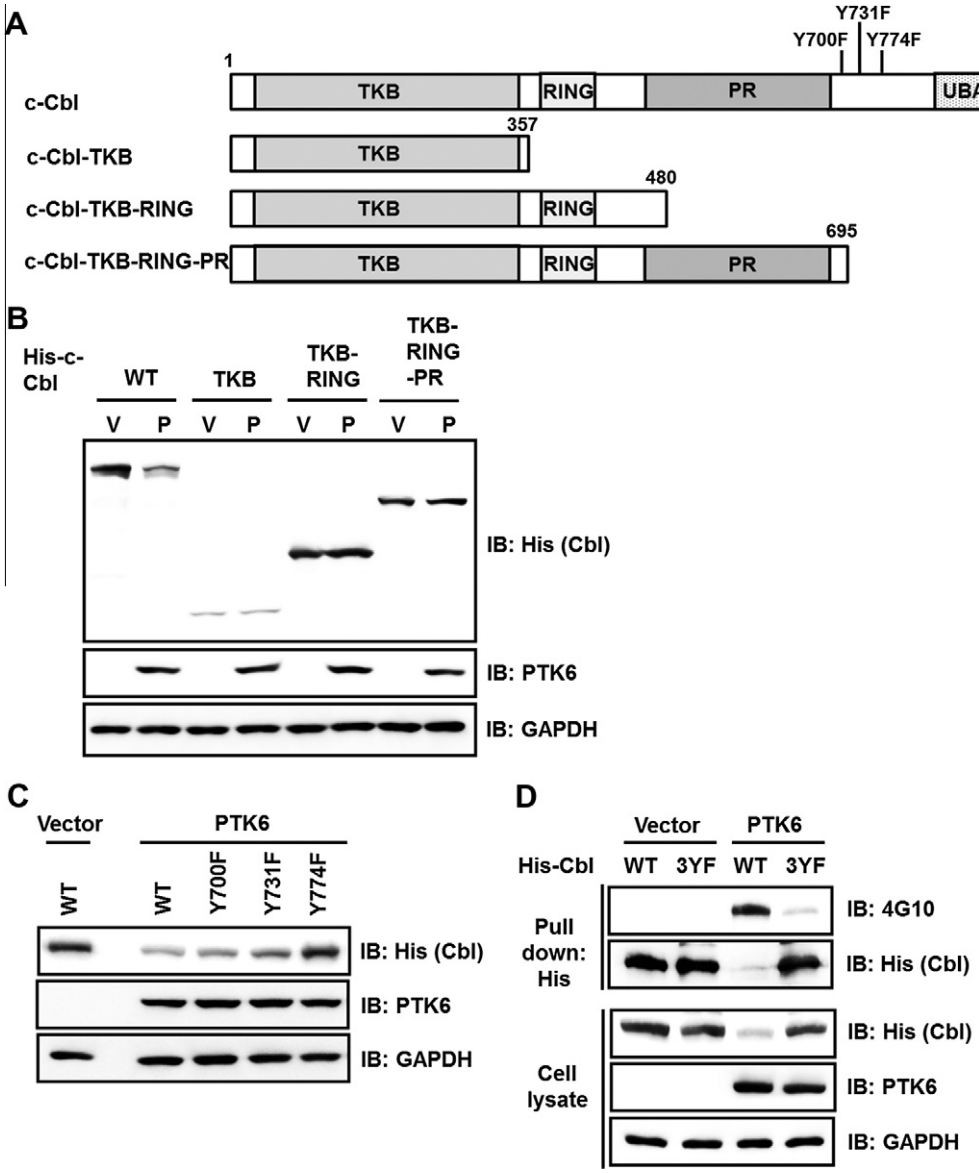

Fig. 3. Determination of PTK6 phosphorylation sites located within c-Cbl, and the effect of phosphorylation-disabling mutations on c-Cbl down-regulation (A) Schematic representation of c-Cbl and its deletion and point mutants. TKB: tyrosine kinase binding domain, RING: RING finger domain, PR: proline-rich domain, UBA: ubiquitin-associated domain. (B) His-c-Cbl or its deletion mutants were transiently expressed in HEK293 cells stably transfected with either empty vector (V) or PTK6 (P). Expression levels of wild-type His-c-Cbl and its deletion mutants were examined by Western blotting with anti-penta-His antibody. (C) Tyr^{700}, Tyr^{731}, and Tyr^{774} residues in the C-terminal domain of c-Cbl were changed to phenylalanine by site-directed mutagenesis. HEK293 cells stably expressing empty vector or PTK6 were transiently transfected with constructs expressing His-c-Cbl, His-c-Cbl-Y700F, His-c-Cbl-Y731F, and His-c-Cbl-Y774F. Expression levels of wild-type His-c-Cbl and its point mutants were examined by Western blotting with anti-penta-His antibody. (D) Phosphorylation of wild-type His-c-Cbl or phosphorylation-defective His-c-Cbl-3YF (Y700F/Y731F/Y774F) mutant was analyzed using a pull-down assay (Ni^{2+}-NTA agarose), and Western blotting using anti-phosphotyrosine antibody.

down-regulation of c-Cbl is dependent on auto-ubiquitination, wild-type HA-c-Cbl or HA-c-Cbl-C381A mutant, which is defective in E3 ligase activity [24], was expressed in HEK293 cells stably expressing either PTK6 or empty vector. Whereas wild-type HA-c-Cbl was degraded upon PTK6 expression, the HA-c-Cbl-C381A mutant was not (Fig. 4B).

In summary, our findings suggest that c-Cbl is phosphorylated by PTK6, resulting in degradation through auto-ubiquitination by its E3 ligase activity.

4. Discussion

The oncoprotein PTK6 is overexpressed in various cancer types. It often functions through enhancement of RTK pathways [4,5], which results in promotion of cell proliferation, survival, migration, and anchorage-independent growth. Herein we propose a novel oncogenic role for PTK6. We demonstrated that PTK6 induces degradation of c-Cbl, a key player in the down-regulation of several oncoproteins.

c-Cbl is a RING finger-dependent E3 ubiquitin ligase. In addition to ubiquitination of its substrates, c-Cbl can auto-ubiquitinate, leading to its degradation [15]. Yokouchi et al. [15] reported that auto-ubiquitinaton of c-Cbl is induced by Src, which they discovered by analysis of a 70Z-Cbl mutant lacking E3 ligase activity. They further found that phosphorylation of the Tyr^{371} residue of c-Cbl by Src is essential to auto-ubiquitination, which suggests that this phosphorylation activity may be the mechanism for Src-induced auto-ubiquitination. However, Bao et al. [25] suggested that phosphorylation of C-terminal tyrosines (Tyr^{700}, Tyr^{731}, and

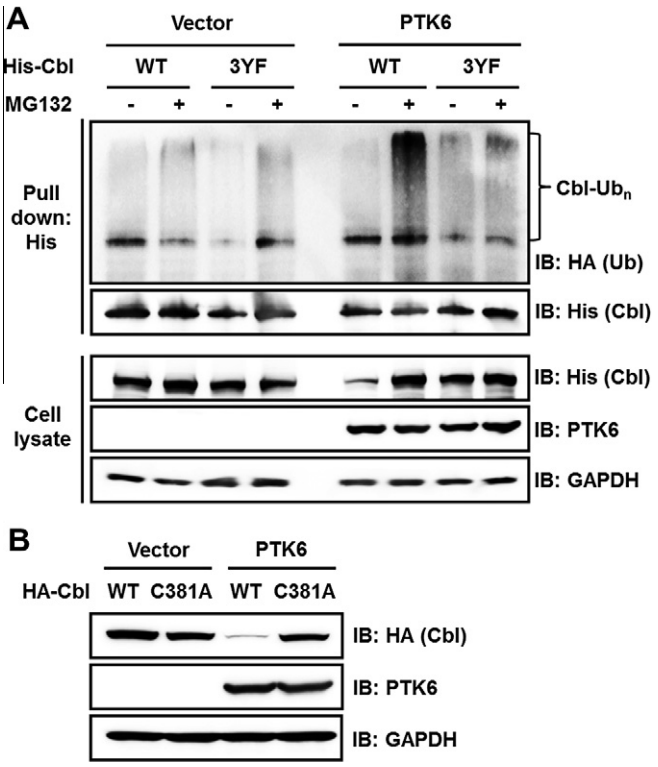

Fig. 4. Analysis of proteasome dependency and auto-ubiquitination during PTK6-induced degradation of c-Cbl. (A) HA-Ubiquitin (Ub) and either His-c-Cbl or His-c-Cbl-3YF, were transiently expressed in HEK293 cells that stably express either empty vector or PTK6. The cells were treated with 20 μM MG132 for 1 h. His-c-Cbl was pulled down using Ni^{2+}-NTA agarose. Ubiquitination of His-c-Cbl or His-c-Cbl-3YF was analyzed by Western blotting using anti-HA antibody. (B) HA-c-Cbl or an E3 ligase-defective HA-c-Cbl-C381A mutant was transiently expressed in HEK293 cells stably expressing either empty vector or PTK6. The level of HA-c-Cbl was examined by Western blotting with anti-HA antibody. GAPDH was used as protein loading control.

Tyr^{774}) of c-Cbl by Src, rather than phosphorylation of Tyr^{371}, is important for its degradation. Moreover, they detected down-regulation of an E3 ligase-defective 70Z-Cbl mutant in Src-expressing cells and therefore propose that c-Cbl does not auto-ubiquitinylate, but rather is ubiquitinated via a different degradation pathway.

In this study, we demonstrated that PTK6 phosphorylates Tyr^{700}, Tyr^{731}, and Tyr^{774} residues in the C-terminal domain of c-Cbl, and that phosphorylation of these tyrosines is important for PTK6-induced down-regulation of c-Cbl. In addition, an E3 ligase-defective mutant, c-Cbl-C381A, was not down-regulated in PTK6-expressing cells whereas the wild-type c-Cbl was. Therefore, our findings clearly demonstrate that PTK6 enhances proteasomal degradation of c-Cbl through phosphorylation of C-terminal tyrosines, which cues auto-ubiquitination of c-Cbl.

c-Cbl negatively regulates RTKs such as the EGF receptor, PDGF receptor, fibroblast growth factor receptor, and c-Met [13,14]. In particular, the EGF receptor is known to be ubiquitinylated by c-Cbl at multiple sites [12]. The ubiquitinated EGF receptor is degraded in lysosomes through clathrin-mediated endocytosis. Previously, we demonstrated that PTK6 induces longer retention of EGF receptor molecules on the cell surface by blocking endocytosis, which signifies that PTK6 effectively enhances EGF receptor signaling [10]. In pursuit of the molecular mechanisms governing this phenomenon, we further demonstrated that PTK6 phosphorylates ARAP1, which contains the ARF-GAP domain, and that the activated ARF-GAP inhibits endocytosis of the surface-bound EGF receptor [10]. Herein we demonstrate a different pathway by which endocytosis of the EGF receptor is attenuated: the PTK6-dependent phosphorylation and consequent proteasomal degradation of c-Cbl. While this study was in progress, Li et al. [10,26] reported that PTK6 inhibits EGF receptor degradation by phosphorylation of the EGF receptor, thus blocking association of the EGF receptor and c-Cbl. Therefore, we suggest that PTK6 negatively affects EGF receptor endocytosis through multiple pathways: through phosphorylation of ARAP1, through phosphorylation and consequent auto-ubiquitination of c-Cbl, and by inhibition of c-Cbl binding to the EGF receptor. In addition to facilitating enhancement of EGF signaling, the PTK6-induced down-regulation of c-Cbl also releases many target molecules of c-Cbl, including RTKs, intracellular PTKs, and other signaling proteins, involved in oncogenic signaling pathways.

In conclusion, our findings provide a novel oncogenic role of PTK6 through promotion of c-Cbl degradation. The findings reported here contribute to understanding the molecular mechanisms of PTK6 as a key effector in tumorigenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.046.

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