学 位 論 文

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Cbl negatively regulates erythropoietin-induced growth and survival signaling through the proteasomal degradation of Src kinase

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Running title: The function of Cbl in erythropoietin signaling

Abstract

We examined the biological functions of the gene Cbl in erythropoietin (EPO) signaling using Cbl-deficient F-36P human erythroleukemia cells by the introduction of the Cbl siRNA expression vector. Knockdown of Cbl promoted EPO-dependent proliferation and survival of F-36P cells, especially at a low concentration of EPO (0.01 U/mL), similar to serum concentrations of EPO in healthy volunteers (0.005-0.04 U/mL). We found that Src was degraded mainly by the proteasomal pathway because the proteasome inhibitor MG-132 but not the lysosome inhibitor NH_4Cl suppressed the EPO-induced degradation of Src in F-36P cells and that knockdown of Cbl inhibited EPO-induced ubiquitination and degradation of Src in F-36P cells. The experiments using the Src inhibitor PP1 and co-expression experiments further confirmed that Cbl and the kinase activity of Src are required for the EPO-induced ubiquitination of Src. In addition, the co-expression experiments and in vitro kinase assay demonstrated that the EPO-induced tyrosine phosphorylation and ubiquitination of Cbl were dependent on the kinase activity of Src but not Jak2. Thus, Cbl negatively regulates EPO signaling mainly through the proteasome-dependent degradation of Src, and the E3 ligase activity of Cbl and its tyrosine phosphorylation are regulated by Src but not Jak2.

Keywords: Cbl; erythropoietin; proteasome; Src; ubiquitination

Introduction

Erythropoietin (EPO) is the primary cytokine that regulates the proliferation and differentiation of erythroid progenitor cells. After the binding of EPO to EPO receptor (EPOR), EPOR is tyrosine-phosphorylated by Janus kinase 2 (Jak2) and the Src family protein tyrosine kinases (PTKs), Src and Lyn, and thereby recruits various signaling molecules to stimulate downstream signaling pathways, including the phosphatidylinositol 3-kinase (PI3-kinase)/Akt and Jak/Stat pathways [1–9].

The c-Cbl proto-oncogenic protein (Cbl) identified as the cellular homologue of v-Cbl contains multiple signaling modules, including a RING domain, an SH2 domain, multiple potential phosphotyrosine residues for SH2-containing proteins, and multiple PXXP docking sites for SH3-containing proteins [10–13]. Thus, Cbl plays several functional roles, acting both as a multivalent adaptor and an E3 ubiquitin-ligase for numerous receptors and non-receptor PTKs, including the Src family PTKs [10–12,14–20]. After cognate ligand stimulation, activated PTKs are ubiquitinated by Cbl and targeted for the 21S proteasome system, resulting in the negative regulation of PTK-mediated signal transduction through the Cbl-dependent degradation of PTKs [12,13,21]. Several studies have shown that activated Src tyrosine-phosphorylates Cbl and activates its ubiquitin ligase activity, resulting in Cbl-dependent ubiquitination and the degradation of Src and Cbl itself [22,23]. Therefore, the dysfunction or impaired recruitment of Cbl to PTKs resulted in deregulated positive signaling that can eventually lead to and contribute to carcinogenesis [24–27].

It was found that EPO induces the tyrosine phosphorylation of Cbl in erythroid cells [28,29]. Cbl was shown to be a negative regulator of developmental and functional properties of multipotent hematopoietic progenitor cells [30]. In addition, Bulut et al. [29] reported that Cbl-deficient erythroid progenitor cells had higher mitogenic activities in response to EPO, and that the p85 regulatory subunit of PI3-kinase ubiquitinated by Cbl recruited an endocytic adaptor protein epsin-1, thereby inducing EPOR internalization and downregulation. These results suggest that Cbl negatively controls EPO signaling through the regulation of EPOR edocytosis. However, the mechanism of the tyrosine phosphorylation of Cbl and its biological functions in

EPOR-mediated signaling remain to be elucidated.

In the present study, we investigated the biological functions of Cbl by using Cbl-deficient F-36P human erythroleukemia cells which were developed by introducing the expression vector for Cbl short interfering RNA (siRNA). We found that the EPO-dependent proliferation and survival of Cbl-deficient F-36P cells was promoted compared to that of mock-transfected F-36P cells, and that Cbl negatively regulates EPOR-mediated signal transduction through the Cbl-dependent ubiquitination and degradation of Src. In addition, Src but not Jak2 regulated the EPO-induced tyrosine phosphorylation and ubiquitination of Cbl.

Materials and Methods

Reagents and plasmids

Recombinant human EPO was kindly provided by the Kirin Brewery Co. (Tokyo, Japan). Cycloheximide, MG-132, PP1, and AG490 were purchased from Calbiochem (San Diego, CA, USA). Anti-Src monoclonal antibody was from Oncogene Science (Cambridge, MA, USA). Anti-actin, anti-Cbl, anti-influenza hemagglutinin (HA), anti-Jak2, anti-Src and anti-ubiquitin polyclonal antibodies and anti-HA, anti-phosphotyrosine (PY) and anti-ubiquitin monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG monoclonal antibody was from Sigma-Aldrich (St Louis, MO). All other agents were purchased from commercial sources.

Expression vectors for the murine wild-type (pUSE-src-wt) and kinase-inactive Src with a mutation of lysine 297 to arginine (pUSE-srcK297R) were purchased from Millipore (Billerica, MA). Expression vectors for the HA-tagged ubiquitin and mouse Jak2 (pSRα-bsr-Jak2) were generous gifts from Dr. D. Bohmann (University of Rochester Medical Center) and Dr. J.N. Ihle (St. Jude Children's Research Hospital), respectively. The expression vector for the human Cbl (pUC-Cbl) was purchased from the American Type Culture Collection (Manassas, VA). The PstI fragment containing full-length Cbl cDNA excised from the pUC vector was blunt-ended by using a DNA blunting kit (Takara, Otsu, Japan). The fragment was subcloned into the EcoRV site of the pUSE plasmid (Upstate, Charlottesville, VA) (pUSE-Cbl). The wild-type, kinase-inactive, and

dominant-negative Src with FLAG epitope tag were generated by PCR-based mutagenesis by fusing the FLAG-sequence (DYKDDDDK) to the C-terminus of those Src cDNAs, as described [31].

Cell culture and establishment of stable transformants of Cbl-deficient F-36P cells

IL-3- and GM-CSF-dependent F-36P human erythroleukemia cells were provided by RIKEN Cell Bank (Tsukuba, Japan). F-36P cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 U/mL human recombinant GM-CSF (the growth medium).

The target sequence for human Cbl is 2342-GAAGCAATGTATAATATTC (the number indicates the location of the first nucleotide of the sequence in the human Cbl coding region). Oligonucleotides containing the siRNA coding sequence were cloned into the BspMI site of plasmid pcPURhU6βicassette (Takara) to generate pCblsiRNA-2342. pCblsiRNA-2342 and the expression vector without any insert were electroporated into F-36P cells using a gene pulser apparatus (Bio-Rad, Hercules, CA) set at 960 μF and 290 V, as described [8,31]. After cells were cultured for 24 h in the growth medium, transformant cells were selected in the growth medium containing 1 μg/mL puromycin (Sigma-Aldrich).

Analyses of cell proliferation and apoptosis

We performed a WST-1 assay to determine cell proliferation according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany), as described [32]. To detect apoptosis, the dual staining of cells with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI) was performed according to the manufacturer's instructions (Roche Diagnostics), as described [32]. Both experiments were repeated independently at least three times.

Transient expression in COS7 and 293T cells

We conducted the transfection of expression plasmids into COS7 and 293T cells using the COSFectin and HEKFectin transfection reagents (Bio-Rad) according

to the manufacturer's instructions. Two days after transfection, the cells were solubilized and subjected to immunoprecipitation, followed by immunoblot analyses.

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting analyses were performed as described [7]. Briefly, F-36P cells pretreated with an inhibitor or a vehicle were stimulated with EPO and lysed with lysis buffer after growth factor starvation by overnight incubation. Cell lysates were then subjected to immunoprecipitation, followed by SDS-PAGE. Separated proteins were electrotransferred to PVDF membranes.

For the immunoblotting analyses, PVDF membranes pre-incubated with 5% bovine serum albumin solution were probed with specific antibodies followed by detection using an enhanced chemiluminescence system (GE Healthcare UK, Buckinghamshire, England).

Analysis of ubiquitination

293T cells transiently transfected with various combination of plasmids or growth factor-starved F-36P were treated with 25 μM of the proteasome inhibitor MG-132 or the same volume of a vehicle as MG-132 for 4 h prior to stimulation with EPO. Those cells were lysed with the lysis buffer containing 10 mM N-ethylmaleimide (Sigma-Aldrich). The lysates were then subjected to immunoprecipitation, followed by immunoblotting analyses as described above.

In vitro kinase assay

The in vitro kinase assay was performed as described [7]. Briefly, anti-Cbl immunoprecipitates from lysates of Cbl-transfected 293T cells were incubated with or without 3 U of affinity-purified recombinant human Src expressed in Sf9 insect cells (Millipore) in a kinase buffer containing 50 μM ATP at 25°C for 10 min. Samples were then subjected to immunoblotting analyses using the indicated antibodies.

Results

Knockdown of Cbl in F-36P cells promotes EPO-induced proliferation of F-36P

cells

To analyze the physiological functions of Cbl in EPOR-mediated signaling, we generated stable transformants of Cbl-deficient F-36P (F-36P-Cbl-siRNA) cells by introducing the expression vector for Cbl siRNA. The expression of Cbl was constitutively suppressed in clones of F-36P-Cbl-siRNA cells (Fig. 1A). First, we analyzed the EPO-dependent proliferation of F-36P-Cbl-siRNA cells by WST-1 assay (Fig. 1B). F-36P-Cbl-siRNA, mock-transfected F-36P (F-36P-mock) and parental cells proliferated dose-dependently of EPO. However, F-36P-Cbl-siRNA cells significantly proliferated more rapidly compared to F-36P-mock and parental cells at lower concentrations of EPO (< 0.1 U/mL). In contrast, the proliferation of those cells was similar at 1 U/mL of EPO.

Knockdown of Cbl prevents F-36P cells from apoptosis

Next, we examined the effects of the knockdown of Cbl expression on the apoptosis of F-36P cells at various concentrations of EPO by flow cytometry using FITC-labeled annexin V. As shown in Table 1, annexin V-positive apoptotic cells were decreased dose-dependently of EPO in both F-36P-Cbl-siRNA and F-36P-mock cells. However, the percentage of annexin V-positive F-36P-Cbl-siRNA cells was lower than that of F-36P-mock cells, especially at a low concentration of EPO (0.01 U/mL) (18.8% vs. 31.7%), similar to serum concentrations of EPO in healthy volunteers (0.005–0.04 U/mL) [33]. The same results were obtained by WST-1 assay using other clones.

The enhancement of proliferation and the prevention of the apoptosis of Cbl-deficient cells were greater compared to those of mock-transfected cells when the concentrations of EPO were reduced, reflecting a hypersensitivity of Cbl-deficient cells to EPO, as described [29].

Src but not Jak2 is involved in the EPO-induced tyrosine phosphorylation of Cbl

The knockdown of Cbl promoted the EPO-dependent proliferation of F-36P cells and prevented the apoptosis of the cells at low concentrations of EPO, similar to the EPO concentrations in the serum of healthy volunteers. To explore the mechanism of Cbl-mediated signal transduction involved in EPO-dependent cell proliferation and survival, we first examined the effects of the Src inhibitor PP1

and Jak2 inhibitor AG490 on the EPO-induced tyrosine phosphorylation of Cbl in F-36P cells.

As shown in Figure 2A, PP1 but not AG490 suppressed the EPO-induced tyrosine phosphorylation of Cbl in F-36P cells. In contrast, AG490 but not PP1 inhibited the EPO-induced tyrosine phosphorylation of a protein band with the MW of 130 kD. These results suggest that Src but not Jak2 is involved in the EPO-induced tyrosine phosphorylation of Cbl.

To further explore the possibility that Src tyrosine-phosphorylates Cbl, Cbl was co-expressed with the wild-type or kinase-inactive Src or wild-type Jak2 in COS7 cells. The wild-type but neither kinase-inactive Src nor the wild-type Jak2 tyrosine-phosphorylated Cbl (Fig. 2B). In addition, the in vitro kinase assay showed that recombinant Src directly tyrosine-phosphorylated Cbl immunoprecipitated from the lysates of Cbl-transfected COS7 cells (Fig. 2C). These findings indicate that Src but not Jak2 is involved in the EPO-induced tyrosine phosphorylation of Cbl.

The knockdown of Cbl reduces the EPO-induced degradation of Src in F-36P cells

It is known that Src is one of the target proteins ubiquitinated by Cbl in the signal transduction pathway mediated by various cytokine receptors and receptor tyrosine kinases [11,12]. To determine whether Cbl is involved in the degradation of Src in EPO signaling, we examined the effects of Cbl on Src expression in F-36P cells after EPO treatment.

The time course analysis showed that the levels of Src expression in F-36P-mock cells were reduced 10 h after EPO treatment (Fig. 3A). In contrast, the levels of Src expression in the Cbl-deficient F-36P cells were not changed 12 h after EPO treatment (Fig. 3B).

Next, we examined the effects of a proteasome inhibitor, MG-132, on the EPO-induced degradation of Src in F-36P cells to determine whether Src is degraded through the proteasomal pathway in EPO signaling. In MG-132-treated cells, a reduction of Src expression was not observed for 12 h, indicating that Src was degraded through the proteasomal pathway in EPO signaling (Fig. 3C). In contrast, the levels of Src expression in F-36P cells

pretreated with the lysosome inhibitor NH_4Cl were similar to those observed in F-36P cells without NH_4Cl pretreatment (Fig. 3D).

These findings indicate that Src is degraded through the proteasomal but not lysosomal pathway and that Cbl plays a crucial role in the proteasomal degradation of Src.

The knockdown of Cbl inhibits the EPO-induced ubiquitination of Src in F-36P cells

Because the proteasomal degradation of proteins requires ubiquitination, we examined the EPO-induced ubiquitination of Src in F-36P cells. As shown in Figure 4A, ubiquitinated Src was detected after EPO treatment in the presence of MG-132 as a high-molecular-weight smear. In addition, Src was slightly ubiquitinated in F-36P cells treated with MG-132 alone.

We next examined the effects of Cbl on the EPO-induced ubiquitination of Src in F-36P cells. The EPO-induced ubiquitination of Src was significantly reduced in Cbl-deficient cells (Fig. 4B), indicating that Cbl plays an important role in the regulation of the EPO-induced ubiquitination of Src.

Src kinase activity and Cbl are required in the EPO-induced ubiquitination of Src

To determine whether the kinase activity of Src is required for its ubiquitination in EPO signaling, we examined the effects of the Src kinase inhibitor PP1 on the EPO-induced ubiquitination of Src in F-36P cells.

As shown in Figure 4C, PP1 dose-dependently suppressed the ubiquitination of Src in EPO-treated F-36P cells. To confirm the findings described above, we performed co-expression experiments using 293T cells (Fig. 4D). Src was ubiquitinated by co-expressing the wild-type Cbl and FLAG-tagged Src. In contrast, Src was not ubiquitinated in the absence of Cbl. In addition, the kinase-inactive and dominant-negative Src was not ubiquitinated in the presence of Cbl. These findings indicated that Cbl is essential for the ubiquitination of Src and that the kinase activity of Src is required for the Cbl-dependent ubiquitination of Src.

The EPO-induced ubiquitination of Cbl is dependent on the kinase activity of Src

but not that of Jak2

We examined the ubiquitination of Cbl induced by EPO in F-36P cells and the effects of PP1 and AG490 on the EPO-induced ubiquitination of Cbl. As shown in Figure 5A,B, PP1 but not AG490 dose-dependently inhibited the EPO-induced ubiquitination of Cbl in F-36P cells. In addition, the results of the co-expression experiments indicated that Cbl was ubiquitinated by the wild-type Src but not by Jak2 or kinase-inactive Src (Fig. 5C). Therefore, the kinase activity of Src but not that of Jak2 is essential for the ubiquitination of Cbl in EPO signaling.

Discussion

The results of the present study demonstrated that Cbl negatively regulates the EPO-dependent proliferation and survival of erythroid cells through the ubiquitination and degradation of Src. In addition, the tyrosine phosphorylation of Cbl and the activation of its ligase activity are dependent on the kinase activity of Src but not that of Jak2 in EPO signaling. The activation of Src is required for the ubiquitination of Src and Cbl.

Our time course analysis demonstrated that the knockdown of Cbl and the proteasome inhibitor MG-132 independently inhibited the EPO-induced degradation of Src. In contrast, the lysosome inhibitor NH_4Cl failed to inhibit the degradation of Src after EPO treatment. Therefore, Src is mainly degraded in the proteasomal but not in the lysosomal pathway, and Cbl plays a crucial role in the degradation of Src in EPO signaling.

Several groups have shown that the Src family PTKs are ubiquitinated by Cbl in PTK- and cytokine receptor-mediated signaling, thereby down-regulating them [11–13]. Yokouchi et al. [22] demonstrated that Src is ubiquitinated by Cbl in the experiments using an in vitro ubiquitination assay. Our observation that EPO failed to induce the ubiquitination of Src in Cbl-deficient F-36P cells indicates that Cbl is required for the EPO-induced ubiquitination of Src. The co-expression experiments using Src and Cbl further confirmed the Cbl-dependent ubiquitination of Src. In addition, because the co-transfection of Cbl with the wild-type Src—but not with the kinase-inactive or dominant-negative Src—induced the ubiquitination of Src, and because PP1 dose-dependently inhibited the EPO-induced ubiquitination of Src, Src kinase activity is required for

the Cbl-dependent ubiquitination of Src, as described [22,23].

A recent study showed that the ubiquitination of an activated Jak2 mutant was enhanced by co-expressing Cbl in 293T cells [34]. However, Cbl is unlikely to be involved in the ubiquitination of Jak2 in EPO signaling because in the present study the EPO-induced ubiquitination of Jak2 was hardly detected in parental F-36P cells, and the wild-type Jak2 was not ubiquitinated when co-expressed with Cbl and ubiquitin in 293T cells (data not shown).

Bulut et al. [29] reported that EPO-induced tyrosine phosphorylation of Cbl is mediated by Jak2 because EPO failed to induce tyrosine phosphorylation of Cbl in γ2A cells expressing HA-tagged EPOR and the kinase-inactive Jak2 and Jak2 directly tyrosine-phosphorylated Cbl in in vitro kinase assays. In contrast, we demonstrated that Cbl was tyrosine-phosphorylated by Src but not Jak2 because PP1 but not AG490 inhibited the EPO-induced tyrosine phosphorylation of Cbl in F-36P cells. The co-expression experiments and the in vitro Src kinase assay further demonstrated that the tyrosine phosphorylation of Cbl is dependent on Src but not Jak2. Although the precise reason is unclear, discrepancy may be related to differences in the parental cells used in both studies. Alternatively, EPO-induced tyrosine phosphorylation of Cbl may be regulated by Jak2 and Src.

The effect of the tyrosine phosphorylation of Cbl on its ligase activity is controversial. Yokouchi et al. [22] showed that the Src-dependent tyrosine phosphorylation of Cbl is necessary for its E3 ligase activity and its own ubiquitination. In contrast, it was found that the Cbl-dependent degradation of Fyn did not require the Fyn-mediated phosphorylation of Cbl [21]. In the present study, the kinase activity of Src but not that of Jak2 is required for the EPO-induced ubiquitination of Cbl as well as Src, because PP1 inhibited the EPO-induced ubiquitination of Cbl and the ubiquitination of Cbl was induced by co-expressing the wild-type but not kinase-inactive Src or the wild-type Jak2. These findings indicate that the Src-dependent tyrosine phosphorylation of Cbl is consistent with the ubiquitination of Cbl by the activation of Src kinase. Therefore, the Src-dependent tyrosine phosphorylation of Cbl may be a trigger that activates its ubiquitin ligase activity, as described [22]. The EPO-induced ubiquitination of Cbl may negatively regulate the Cbl-dependent ubiquitination

and degradation of Src that transduces growth and survival signals mediated by EPOR.

Conclusion

The Cbl-dependent ubiquitination and degradation of Src plays an important role in the EPO-dependent proliferation and survival of erythroid cells. The E3 ligase activity of Cbl and its tyrosine phosphorylation are regulated by Src but not Jak2. In addition, because Cbl appears to be ubiquitinated by itself in EPO signaling, the ubiquitination of Cbl may control its inhibitory effects on Src-mediated EPO signaling. Further experiments are required, however, to determine the signaling molecules functioning downstream of Cbl in the signal transduction of EPO-induced proliferation and survival of erythroid cells.

Contributions

T.S., F.O.W., and Y.K. designed and performed the research, analyzed the data and wrote the paper; A.K. analyzed the data and commented on the paper; T.T. supervised the research and commented on the paper. All authors have approved the final version.

Conflict-of-interest-disclosure

All authors have no conflict of interest to declare.

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Figure legends

Fig. 1. Knockdown of Cbl enhances the EPO-dependent proliferation of F-36P cells. (A) The transformant cells of Cbl-deficient and mock-transfected F-36P cells were established by introducing the expression vector for Cbl siRNA as described in Materials and Methods (F-36P-Cbl-siRNA and F-36P-mock cells, respectively). Lysates from F-36P-Cbl-siRNA, F-36P-mock, and parental F-36P cells were subjected to immunoprecipitation with anti-Cbl antibody followed by a Western blotting analysis using anti-Cbl antibody. (B) F-36P-Cbl-siRNA and F-36P-mock cells were cultured with increasing concentrations of EPO for 4 days. The proliferation of those cells was then analyzed by WST-1 assay. The data are mean ± standard deviation (SD) of the absorbance at 450 nm. (1) F-36P-Cbl-siRNA (1-C2) cells, (2) F-36P-Cbl-siRNA (1-B2) cells, (3) F-36P-mock cells, (4) F-36P-parental cells.

Fig. 2. Src but not Jak2 is involved in the EPO-induced tyrosine phosphorylation of Cbl. (A) The effects of protein tyrosine kinase (PTK) inhibitors on the EPO-induced tyrosine phosphorylation of Cbl. F-36P cells were serum- and cytokine-starved for 16 h. The cells were then treated with the indicated concentrations of PP1 or AG490 for 1 h or 16 h, respectively, followed by stimulation with 100 U/mL of EPO at 37°C for 10 min. Cells treated with 0.1% DMSO were used as a control. The lysates were subjected to immunoprecipitation with anti-Cbl antibody. Immunoprecipitated proteins were subjected to SDS-PAGE, and separated proteins were electrotransferred to PVDF membranes. For the Western blotting analysis, the membranes pre-incubated with 5% bovine serum albumin solution were probed with anti-phosphotyrosine (PY) antibody, and then reprobed with anti-Cbl antibody. The protein bands were visualized with a chemiluminescence system. (B) Src but not Jak2 tyrosine-phosphorylates Cbl in COS7 cells. The expression vectors were transfected into COS7 cells in the indicated combinations. Cell lysates were immunoprecipitated with anti-Cbl antibody and analyzed by Western blotting analyses using anti-PY antibody. The membranes were reprobed with the indicated antibodies. WT; wild-type, KD; kinase-inactive. WCL, whole cell lysates. (C) The in vitro tyrosine phosphorylation of Cbl by Src. Anti-Cbl

immunoprecipitates from the lysates of COS7 cells transfected with Cbl alone were incubated with or without 3 U of affinity-purified recombinant human Src (Src-WT) expressed in Sf9 insect cells in a kinase buffer containing 50 μM ATP at 25°C for 10 min. The reaction was stopped, and the samples were subjected to Western blotting analyses using the indicated antibodies.

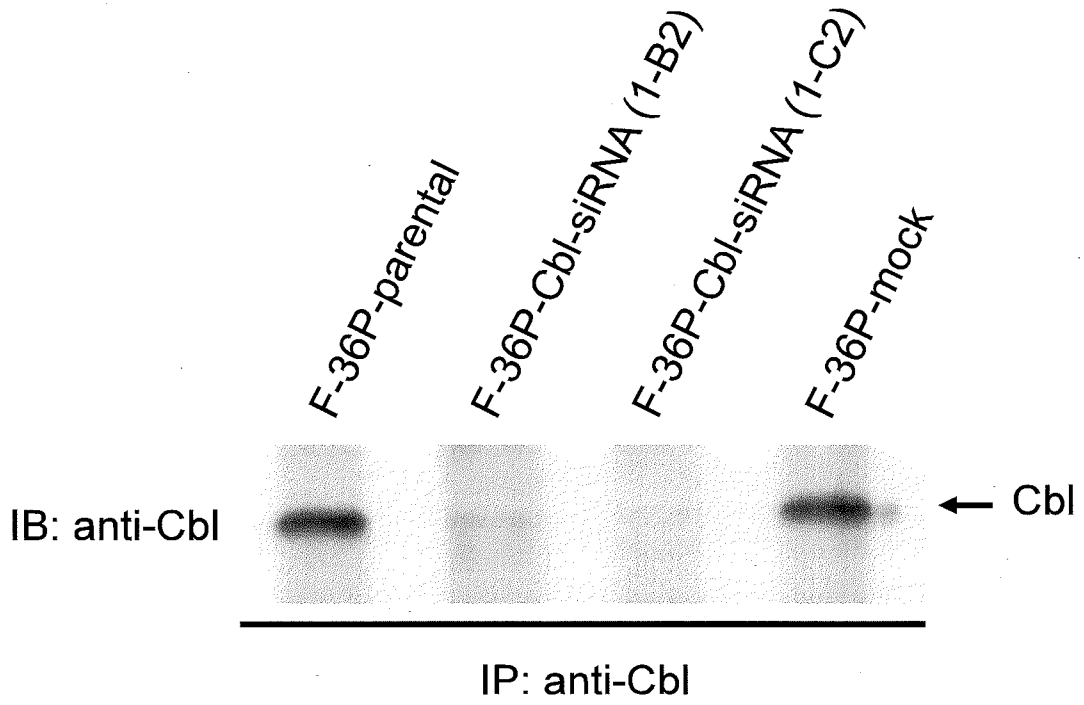
Fig. 3. The Cbl-dependent degradation of Src by the proteasomal pathway in EPO signaling. After growth factor starvation, parental, Cbl-deficient and mock-transfected F-36P (F-36P-Cbl-siRNA and F-36P-mock, respectively) cells were treated with 10 μM cycloheximide (CHX) at 37°C for 1 h in conjunction with or without 25 μM MG-132 or 20 mM NH_4Cl. The cells were then stimulated with 100 U/mL EPO at 37°C for the indicated times. Cell lysates were immunoprecipitated with anti-Src antibody followed by Western blotting analysis using anti-Src antibody. A Western blotting analysis using anti-β-actin antibody was also performed (WCL, whole cell lysates). The data shown are representative of three independent experiments. The results of the time course analysis in mock-transfected F-36P cells were similar to those in parental cells. (A) F-36P-mock cells treated with CHX alone, (B) F-36P-Cbl-siRNA cells treated with CHX alone, (C) F-36P-mock cells treated with CHX and MG-132, (D) F-36P-mock cells treated with CHX and NH_4Cl.

Fig. 4. The kinase activity of Src is required for the Cbl-dependent ubiquitination of Src. (A) The EPO-induced ubiquitination of Src in F-36P cells. After growth factor starvation, F-36P cells were treated with 25 μM MG-132 at 37°C for 4 h prior to EPO (100 U/mL) stimulation at 37°C for 10 min. Cell lysates were then immunoprecipitated with anti-Src antibody followed by Western blotting analyses using the indicated antibodies. Ubi; ubiquitin. (B) The knockdown of Cbl inhibits the EPO-induced ubiquitination of Src in F-36P cells. Cbl-deficient and mock-transfected F-36P cells were treated with 25 μM MG-132 at 37°C for 4 h prior to EPO stimulation as described above. Cell lysates were then immunoprecipitated with anti-Src antibody followed by Western blotting analyses using the indicated antibodies. (C) Inhibitory effects of PP1 on the EPO-induced ubiquitination of Src in F-36P cells. After growth factor-starved F-36P cells were

treated with 25 μM MG-132 at 37°C for 3 h, the cells were treated with PP1 at the indicated concentrations for more 1 h prior to EPO (100 U/mL) stimulation at 37°C for 10 min. Cell lysates were then immunoprecipitated with anti-Src antibody followed by Western blotting analyses using the indicated antibodies. (D) The co-expression experiments using Src and Cbl. Cbl was transiently expressed with the wild-type, kinase-inactive, or dominant-negative FLAG-tagged Src and HA-tagged ubiquitin in 293T cells. Two days after transfection, cell lysates were prepared. The lysates were immunoprecipitated with anti-FLAG antibody followed by Western blotting analyses using the indicated antibodies. HA; influenza hemagglutinin, WCL; whole cell lysates.

Fig. 5. The kinase activity of Src but not that of Jak2 is required for the EPO-induced ubiquitination of Cbl. (A) Inhibitory effects of PP1 on EPO-induced ubiquitination of Cbl in F-36P cells. After growth factor-starved F-36P cells were treated with 25 μM MG-132 at 37°C for 3 h, the cells were treated with PP1 at the indicated concentrations at 37°C for more 1 h prior to EPO (100 U/mL) stimulation at 37°C for 10 min. Cell lysates were then immunoprecipitated with anti-Cbl antibody followed by Western blotting analyses using the indicated antibodies. Ubi; ubiquitin. (B) Effects of AG490 on the EPO-induced ubiquitination of Cbl. After growth factor-starved F-36P cells were treated with 100 μM AG490 at 37°C for 12 h, the cells were treated with 25 μM MG-132 at 37°C for more 4 h. The cells were then treated with EPO (100 U/mL) stimulation at 37°C for 10 min. Cell lysates were immunoprecipitated with anti-Cbl antibody followed by Western blotting analyses using the indicated antibodies. (C) Src but not Jak2 induced the ubiquitination of Cbl in 293T cells. Cbl was transiently transfected with HA-tagged ubiquitin and the wild-type and kinase-inactive Src and wild-type Jak2 using HEKFectin transfection reagents. Two days after transfection, cell lysates were immunoprecipitated with anti-Cbl antibody followed by Western blotting analyses using the indicated antibodies. HA; influenza hemagglutinin.

Fig. 1A


F-36P-parental
F-36P-Cbl-siRNA (1-B2)
F-36P-Cbl-siRNA (1-C2)
F-36P-mock
IB: anti-Cbl
Cbl
IP: anti-Cbl

Figure

Fig. 1B

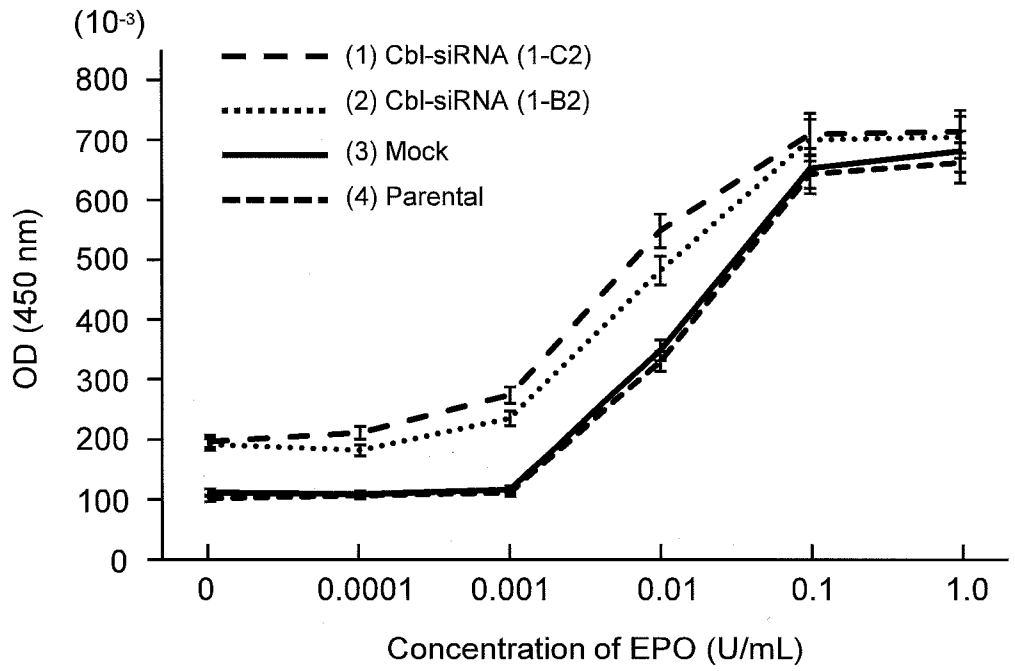
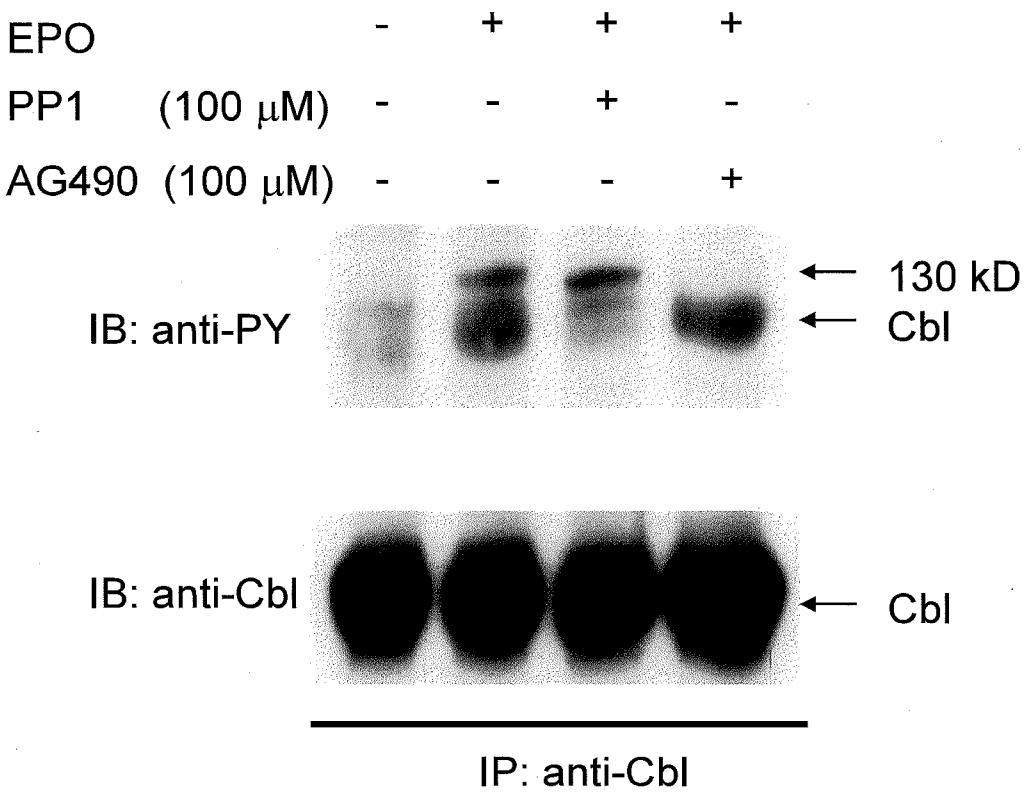


Fig. 2A


EPO - + + +
PP1 (100 μM) - - + -
AG490 (100 μM) - - - +
IB: anti-PY
130 kD
Cbl
IB: anti-Cbl
Cbl
IP: anti-Cbl

Figure

Fig. 2B

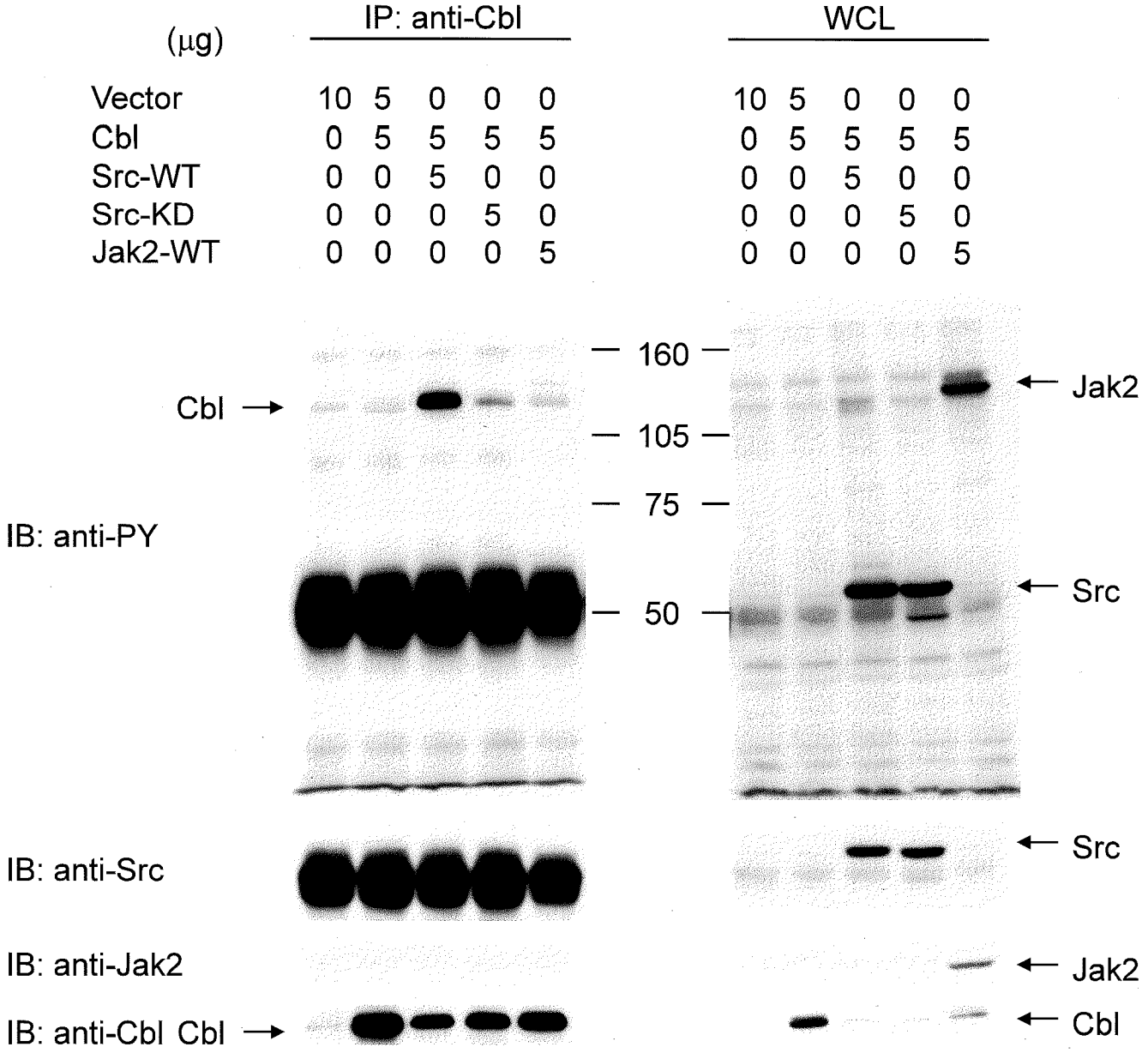


Fig. 2C

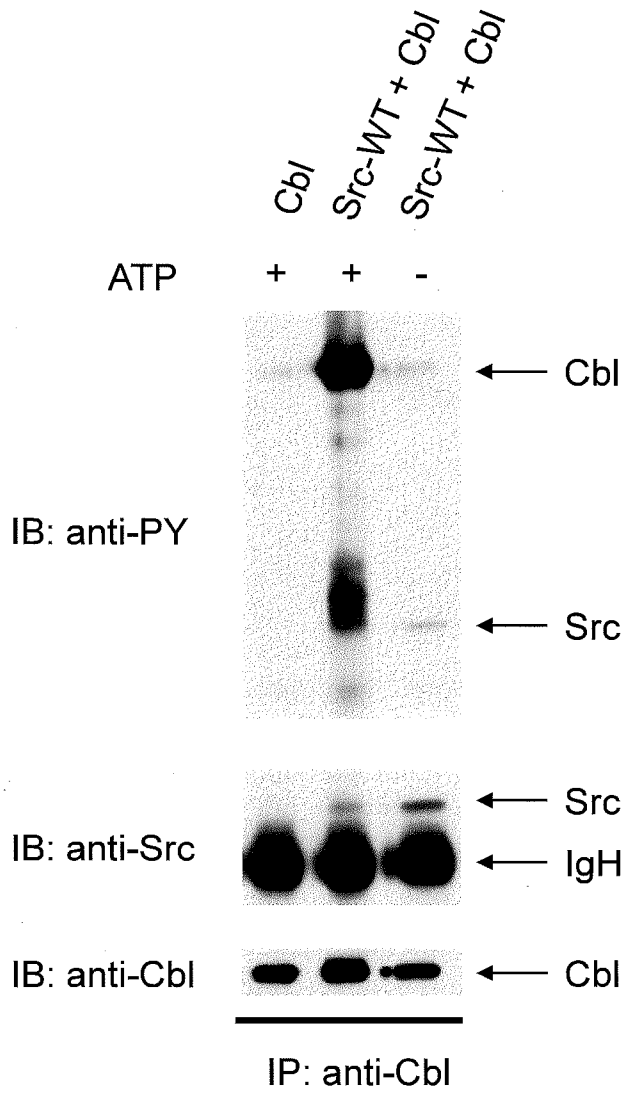

Cbl
Src-WT + Cbl
Src-WT + Cbl
ATP
+
+
-
IB: anti-PY
Cbl
Src
IB: anti-Src
Src
IgH
IB: anti-Cbl
Cbl
IP: anti-Cbl

Fig. 3A

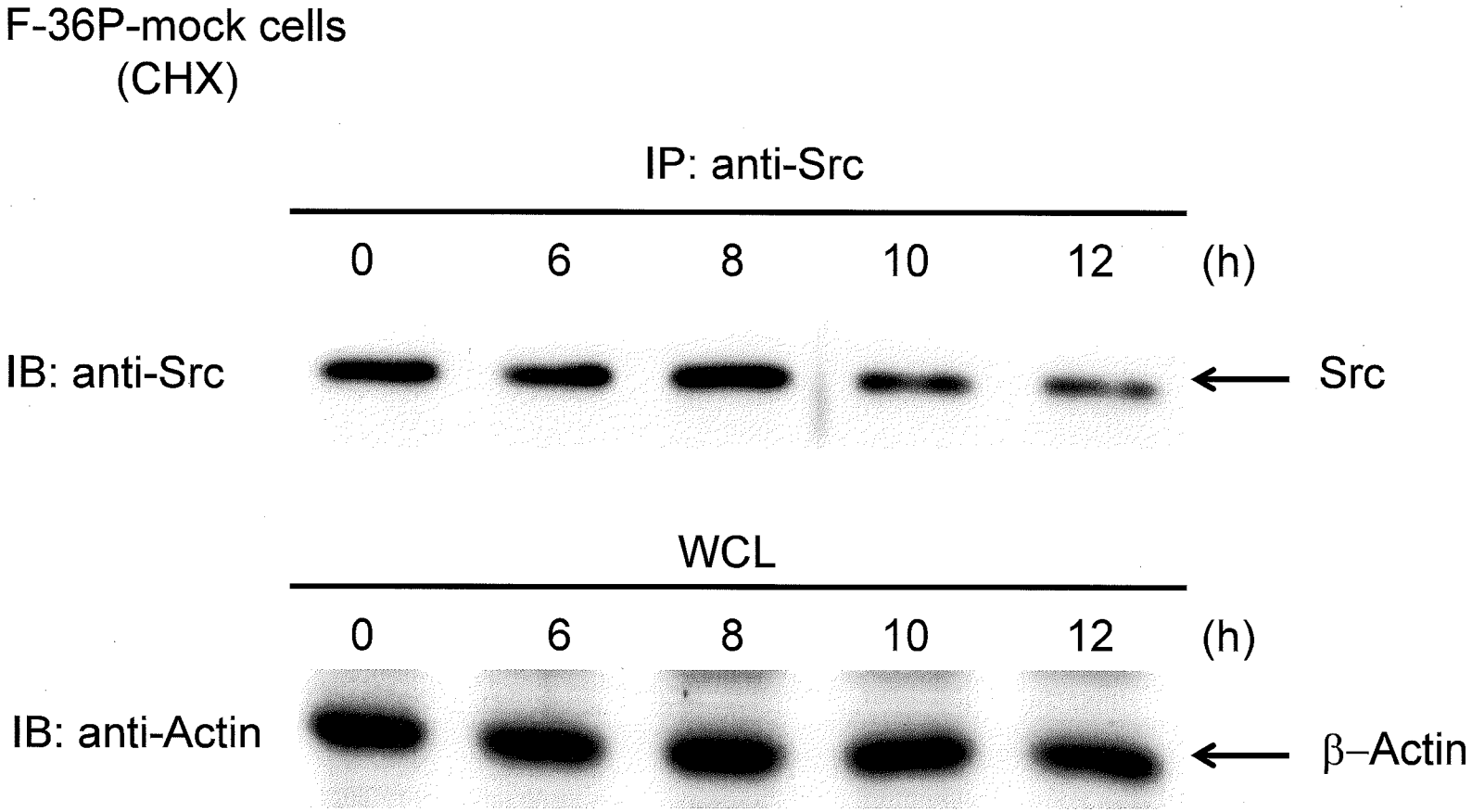
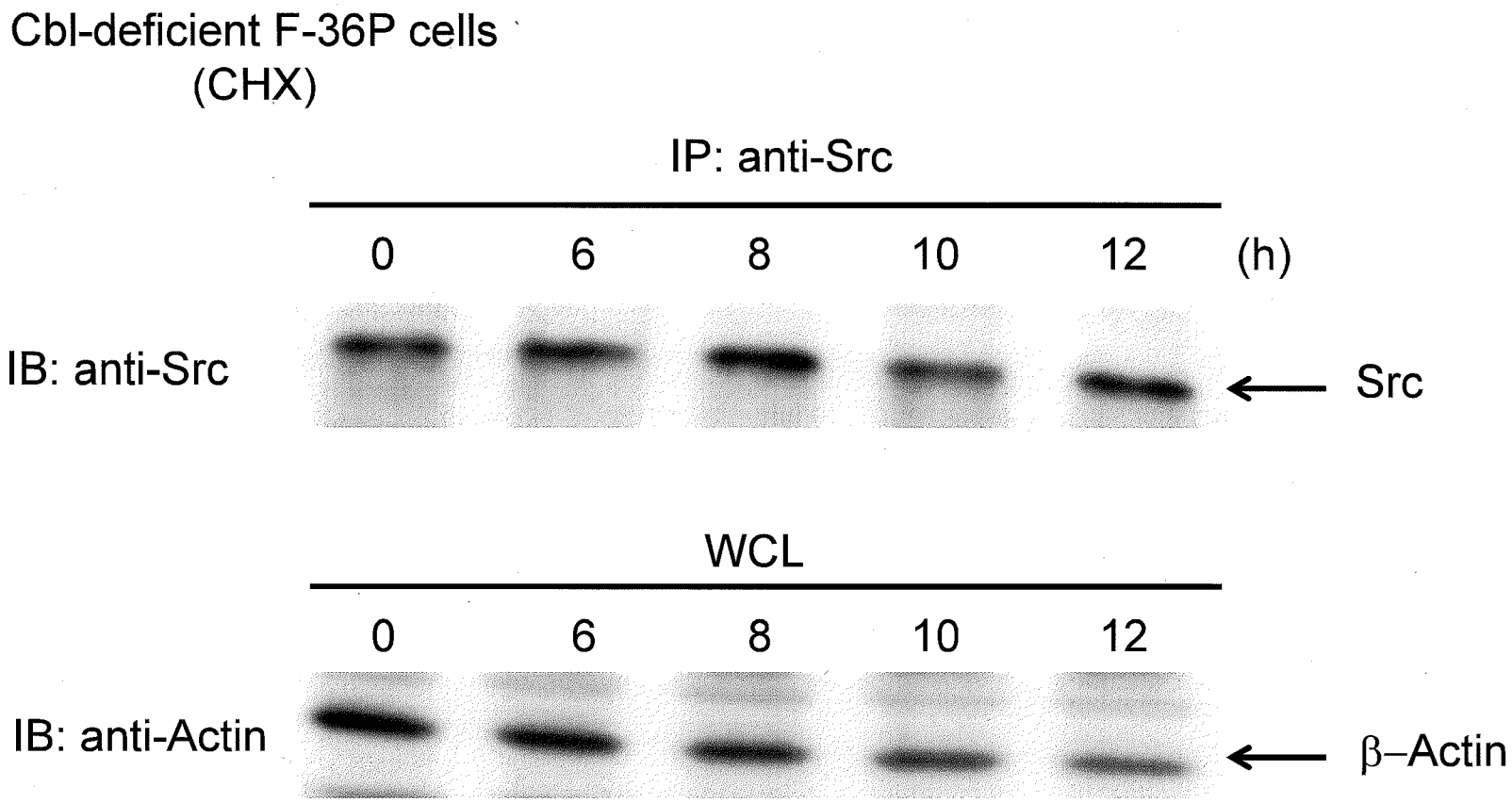

F-36P-mock cells
(CHX)
IP: anti-Src
0
6
8
10
12
(h)
IB: anti-Src
Src
WCL
0
6
8
10
12
(h)
IB: anti-Actin
β–Actin

Fig. 3B


Cbl-deficient F-36P cells
(CHX)
IP: anti-Src
0
6
8
10
12
(h)
IB: anti-Src
Src
WCL
0
6
8
10
12
IB: anti-Actin
β–Actin

Figure

Fig. 3C

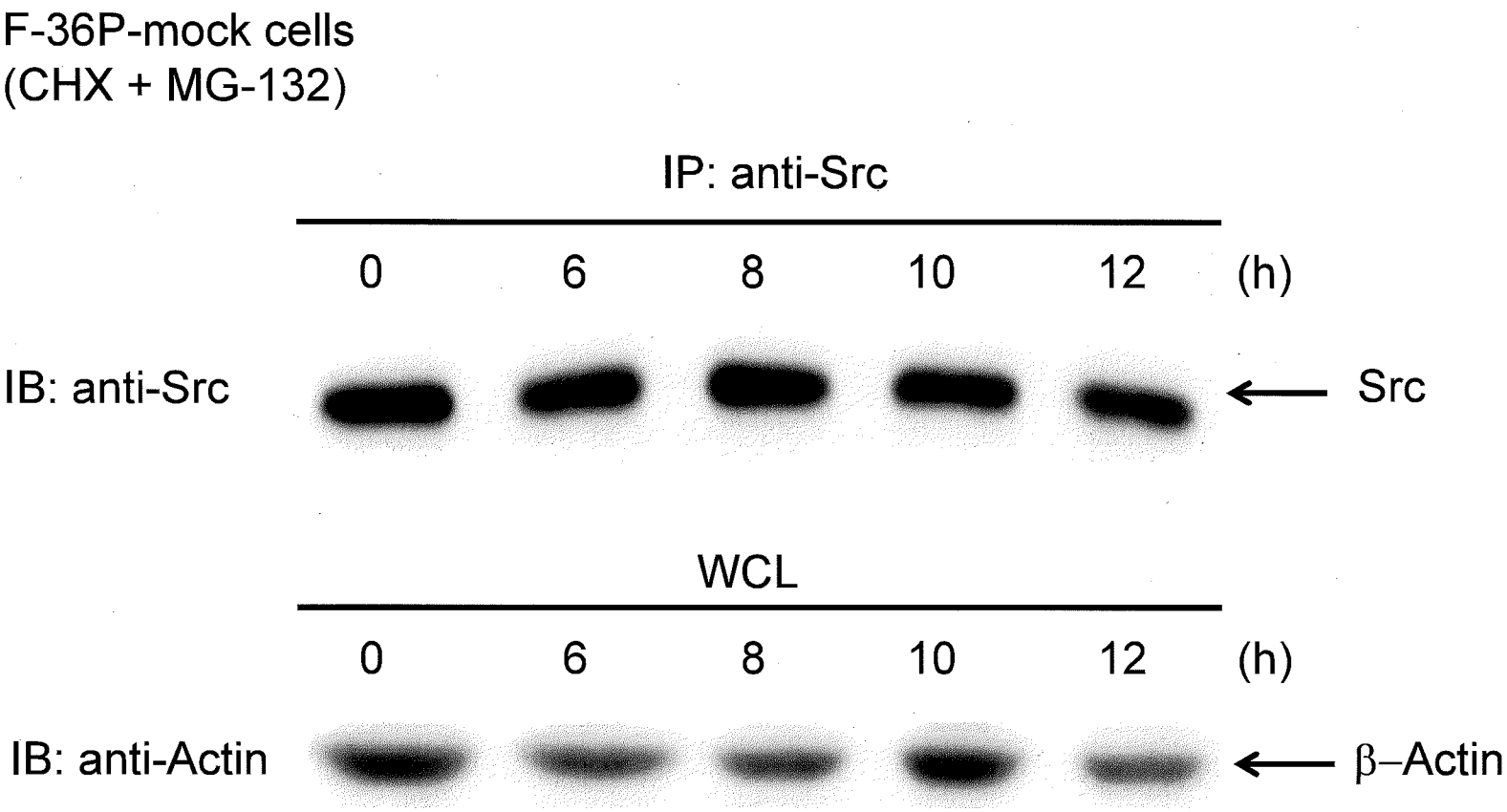
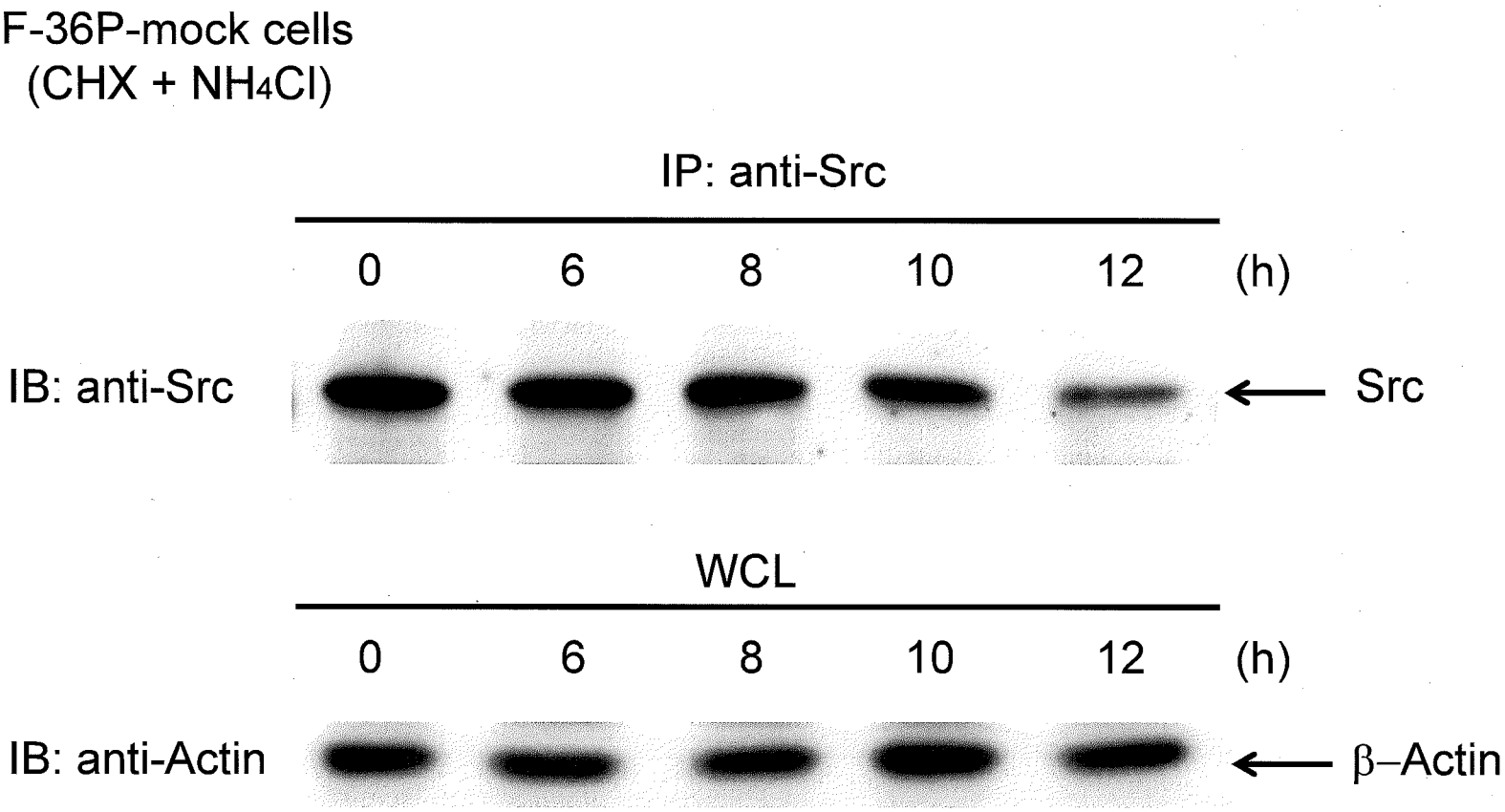


Fig. 3D


F-36P-mock cells
(CHX + NH4Cl)
IP: anti-Src
0
6
8
10
12
(h)
IB: anti-Src
Src
WCL
0
6
8
10
12
(h)
IB: anti-Actin
β–Actin

Figure

Fig. 4A

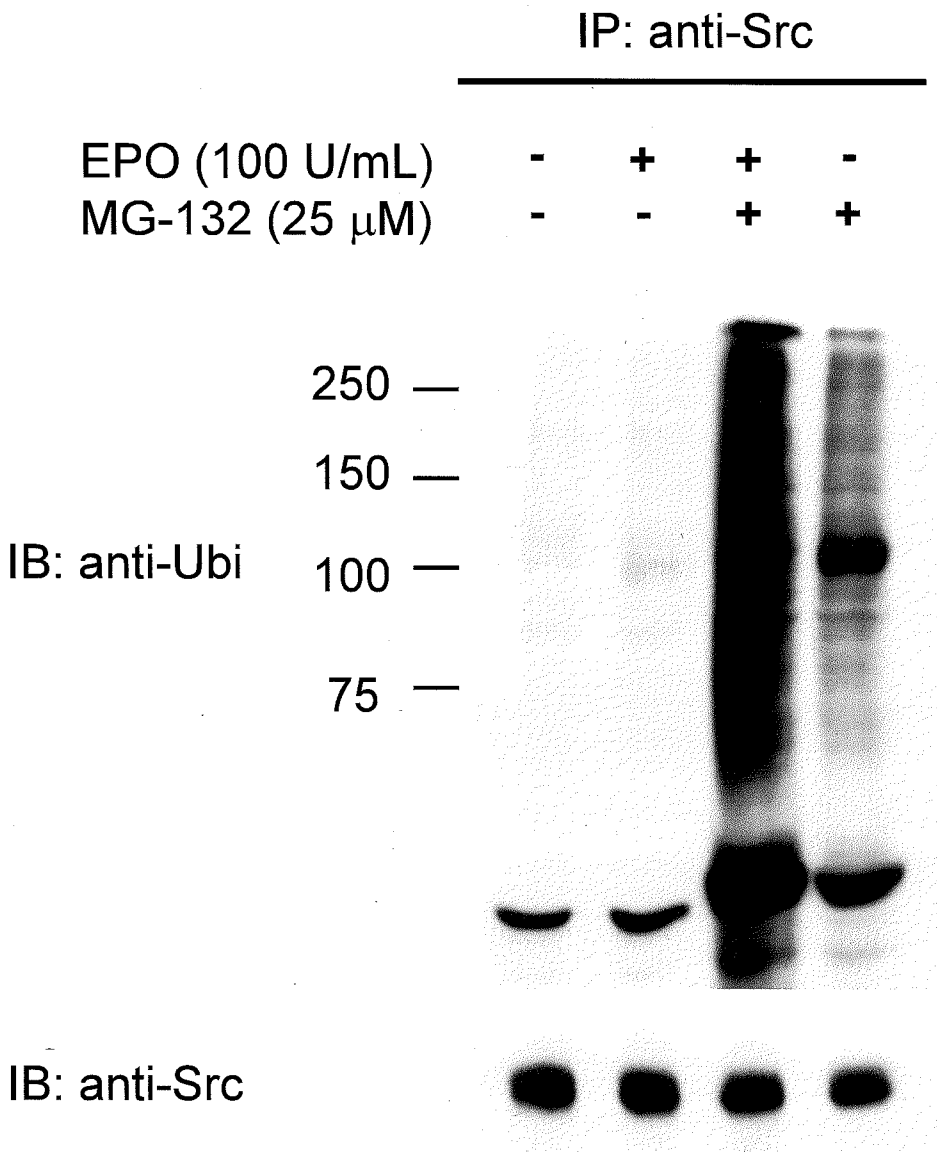
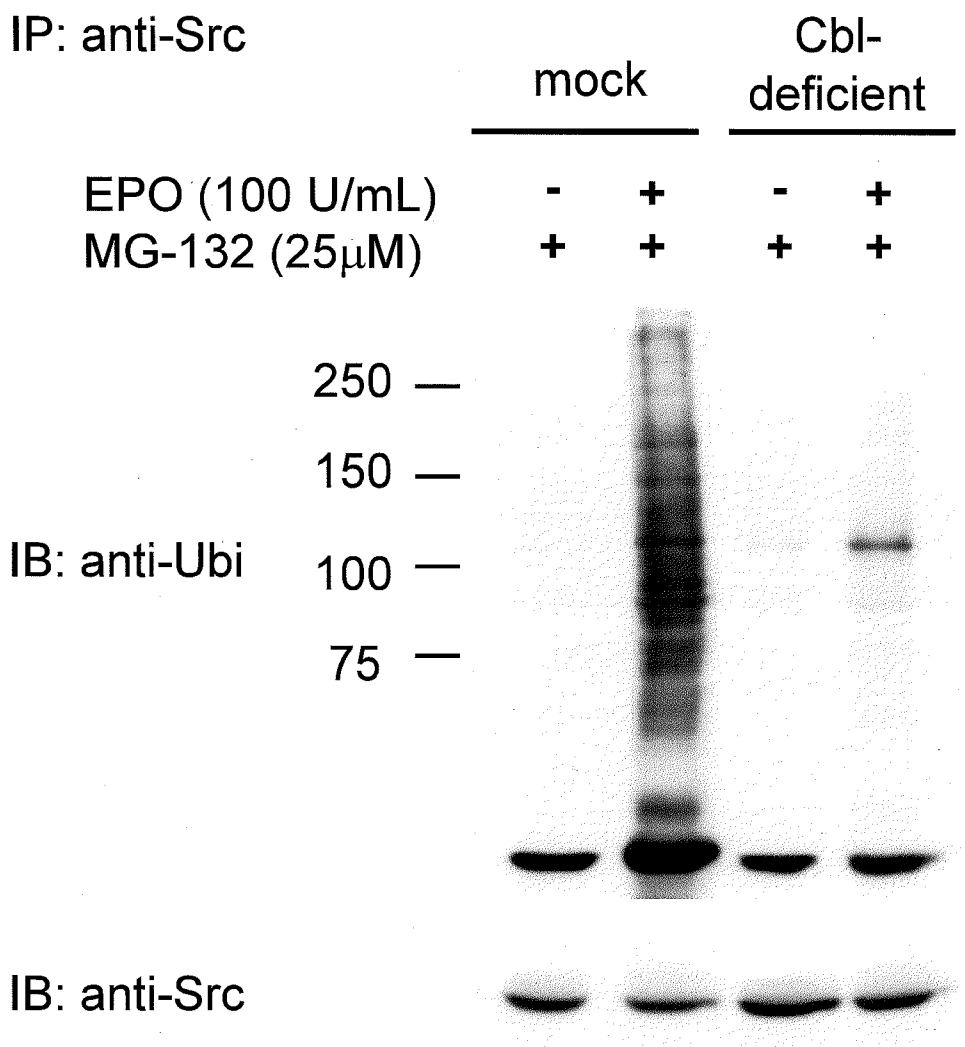


Fig. 4B


IP: anti-Src
mock
Cbl-deficient
EPO (100 U/mL)
MG-132 (25μM)
- + - +
+ + + +
250
150
100
75
IB: anti-Ubi
IB: anti-Src

Figure

Fig. 4C

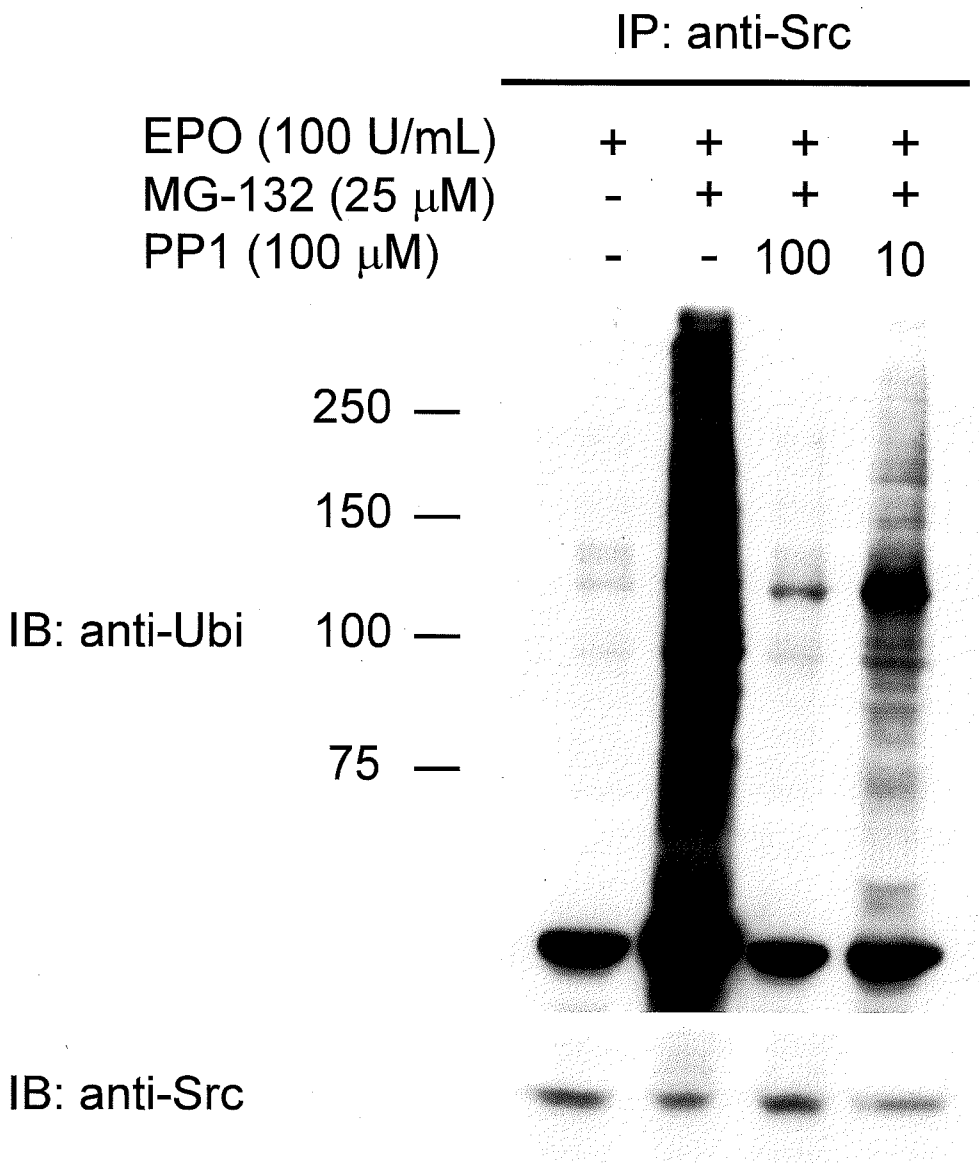


Fig. 4D

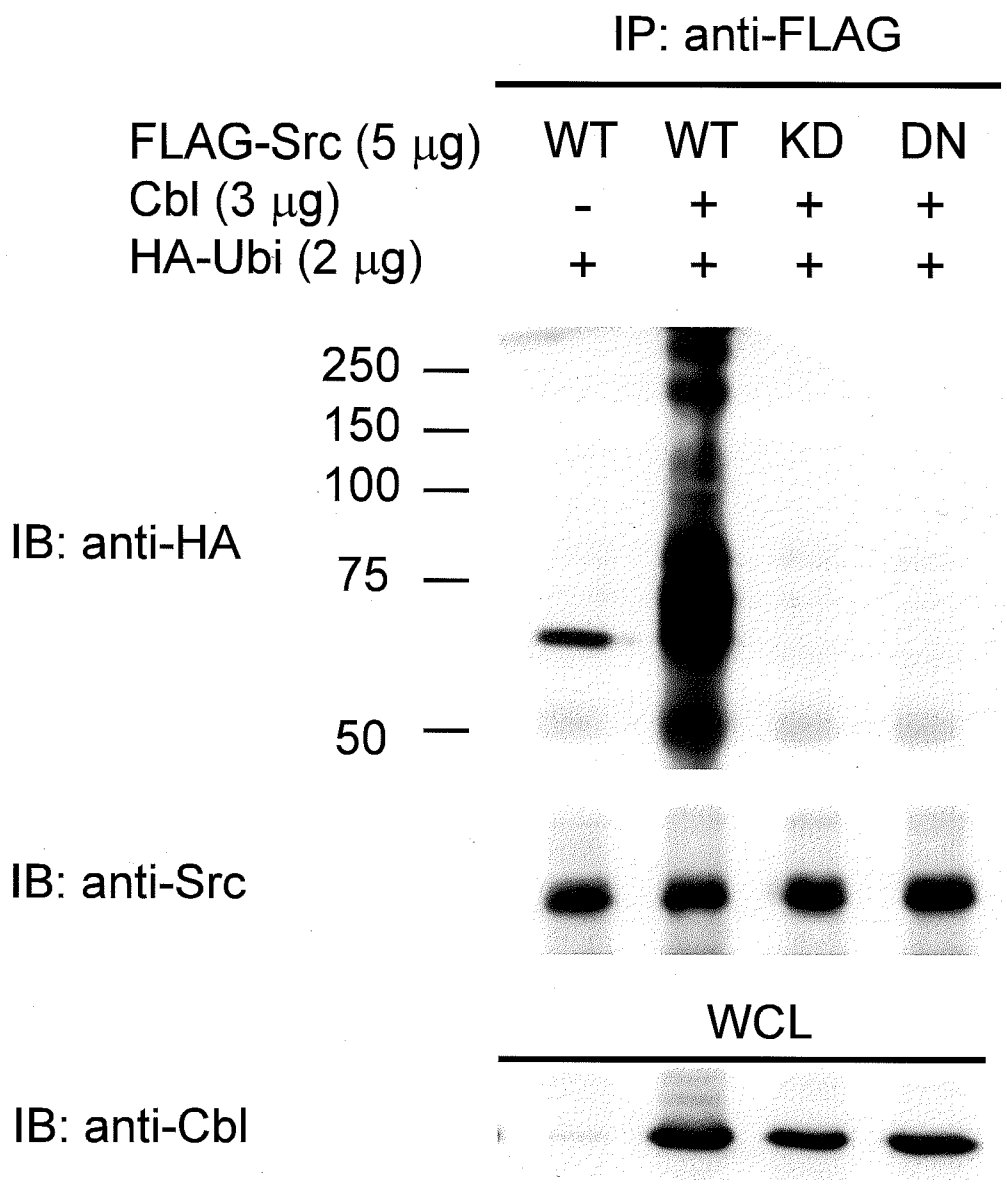
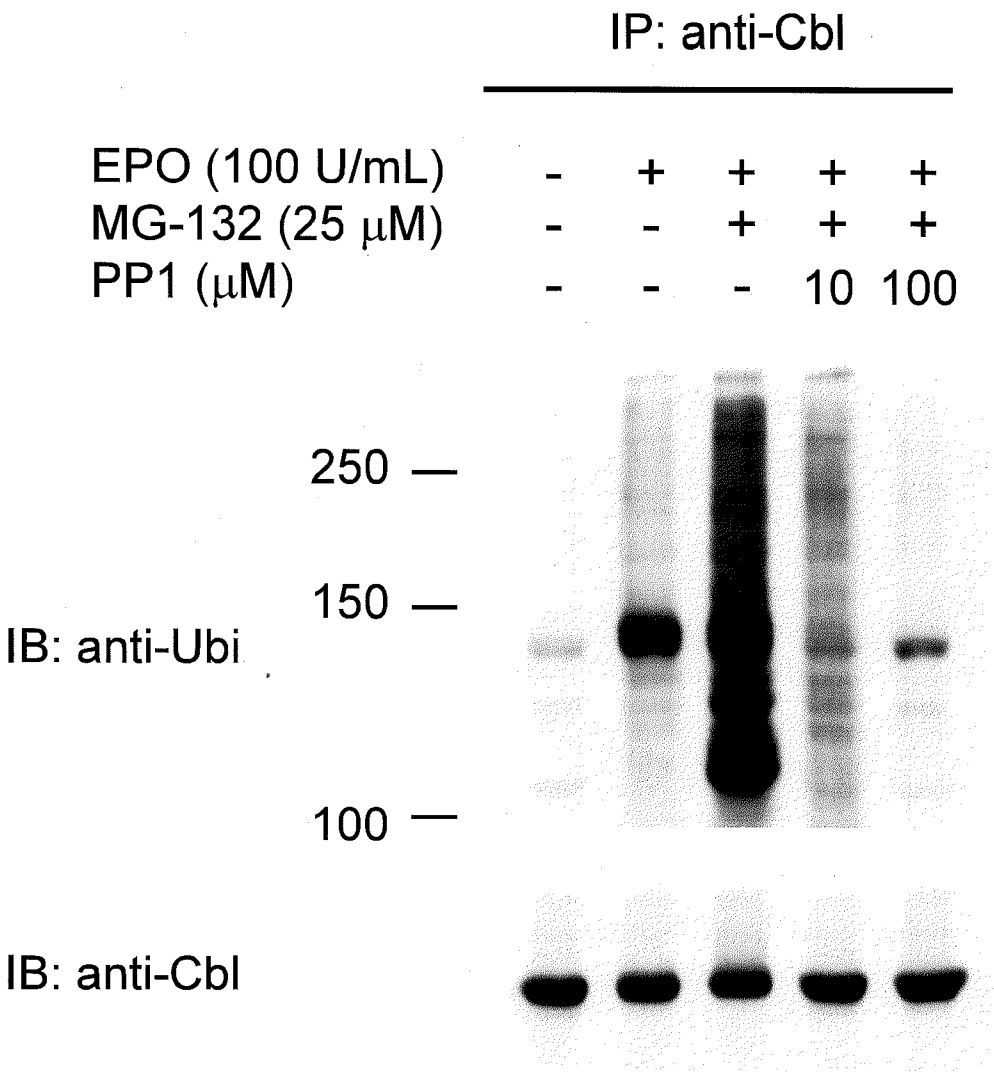
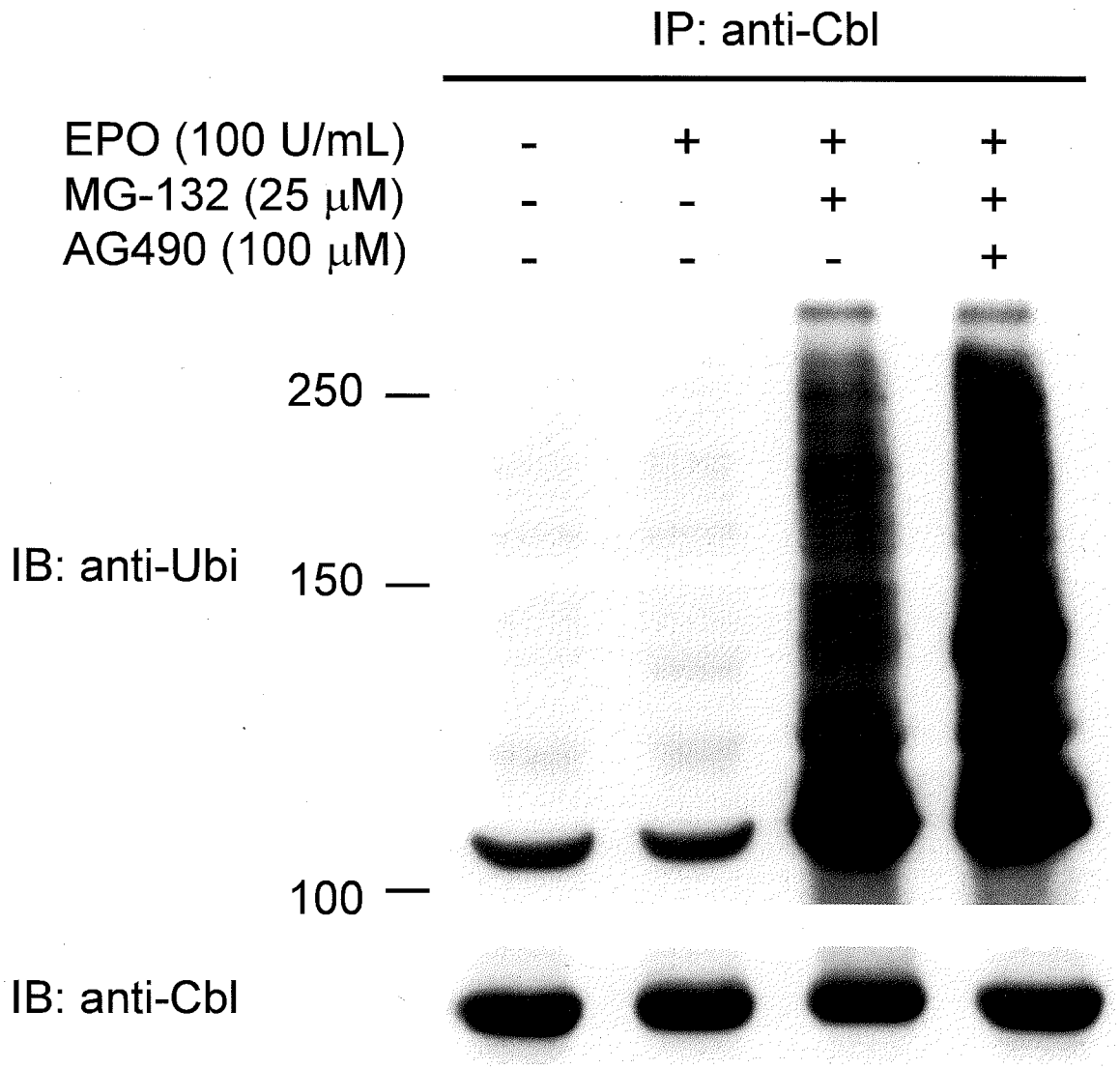

IP: anti-FLAG
FLAG-Src (5 μg) WT WT KD DN
Cbl (3 μg) - + + +
HA-Ubi (2 μg) + + + +
250
150
100
75
50
IB: anti-HA
IB: anti-Src
WCL
IB: anti-Cbl

Fig. 5A


IP: anti-Cbl
EPO (100 U/mL) - + + + +
MG-132 (25 μM) - - + + +
PP1 (μM) - - - 10 100
250
150
100
IB: anti-Ubi
IB: anti-Cbl

Figure

Fig. 5B



Figure

Fig. 5C

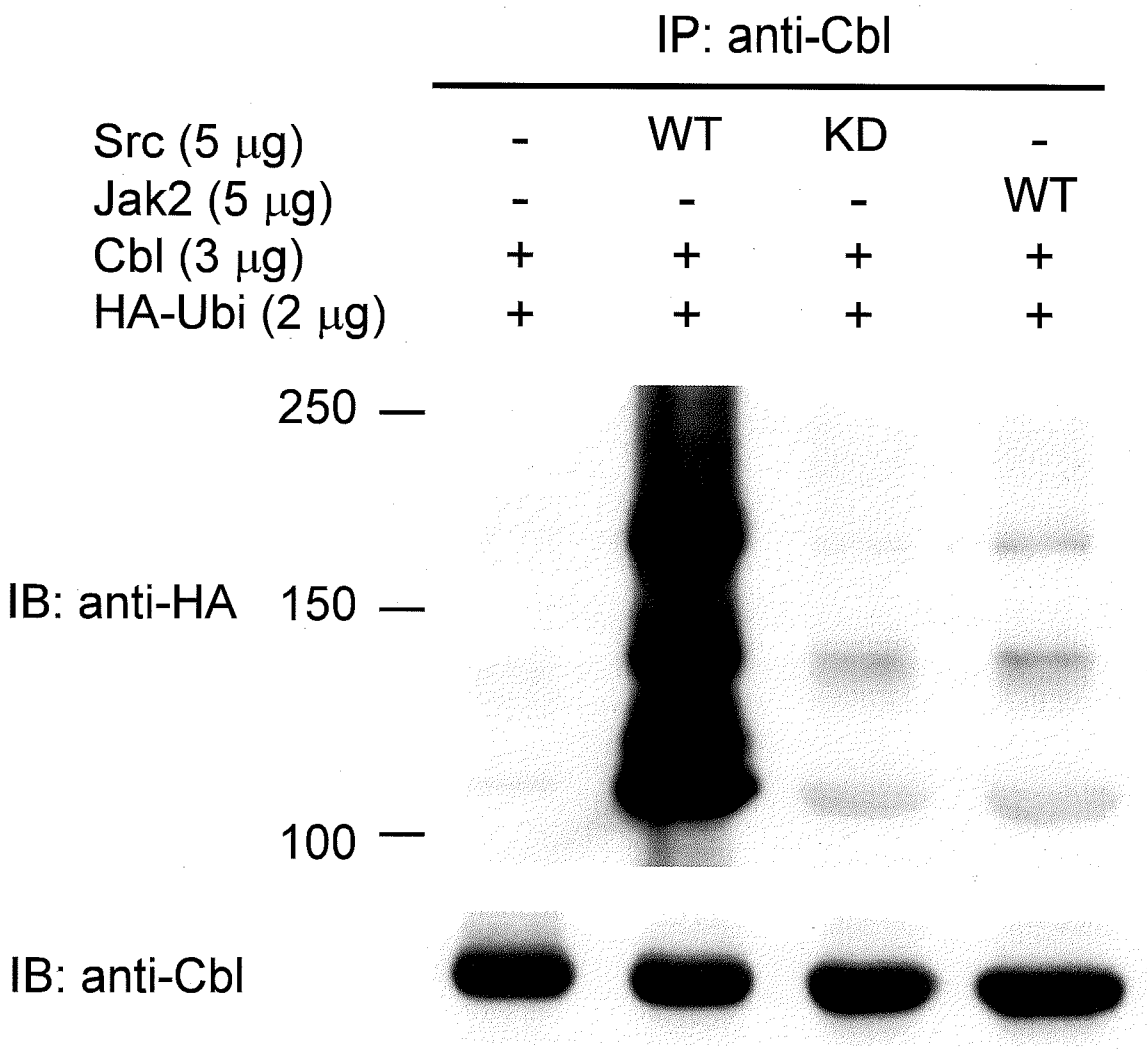


Table 1.

Knockdown of Cbl prevents F-36P cells from apoptosis.

	EPO (U/mL)		0	0.01	0.1	1
Mock	A (+)	(%)	41.4	31.7	14.9	13.5
	A (-) PI (+)	(%)	15.8	3.9	3.6	3.4
Cbl-siRNA	A (+)	(%)	30.2	18.8	10.1	10.7
	A (-) PI (+)	(%)	3.7	2.6	3.9	3.7

After F-36P-Cbl-siRNA (Cbl-siRNA) and F-36P-mock (mock) cells were cultured with increasing concentrations of EPO for 3 days, the cells were stained with FITC-labeled annexin V and propidium iodide (PI). The cells were then analyzed with an EPICS XL flow cytometry system equipped with EXPO32ADC software (Beckman Coulter, Miami, FL). The data represent the means of three independent experiments. The data represent the percentage of annexin V-positive [A (+)] or annexin V-negative and PI-positive [A (-) PI (+)] cells.