Cbl-mediated Negative Regulation of Platelet-derived Growth Factor Receptor-dependent Cell Proliferation

A CRITICAL ROLE FOR Cbl TYROSINE KINASE-BINDING DOMAIN*

(Received for publication, December 14, 1998, and in revised form, April 2, 1999)

Sachiko Miyake‡, Karen P. Mullan-Robinson§, Nancy L. Lill¶, Patrice Douillard*i, and Hamid Band**

From the Lymphocyte Biology Section, Division of Rheumatology, Immunology and Allergy, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

The Cbl proto-oncogene product has emerged as a novel negative regulator of receptor and non-receptor tyrosine kinases. Our previous observations that Cbl overexpression in NIH3T3 cells enhanced the ubiquitination and degradation of the platelet-derived growth factor receptor-α (PDGFRα) and that the expression of oncogenic Cbl mutants up-regulated the PDGFRα signaling machinery strongly suggested that Cbl negatively regulates PDGFRα signaling. Here, we show that, similar to PDGFRα, selective stimulation of PDGFRβ induces Cbl phosphorylation, and its physical association with the receptor. Overexpression of wild type Cbl in NIH3T3 cells led to an enhancement of the ligand-dependent ubiquitination and subsequent degradation of the PDGFRβ, as observed with PDGFRα. We show that Cbl-dependent negative regulation of PDGFRα and β results in a reduction of PDGF-induced cell proliferation and protection against apoptosis. A point mutation (G306E) that inactivates the tyrosine kinase binding domain in the N-terminal transforming region of Cbl compromised the PDGF-inducible tyrosine phosphorylation of Cbl although this mutant could still associate with the PDGFR. More importantly, the G306E mutation abrogated the ability of Cbl to enhance the ligand-induced ubiquitination and degradation of the PDGFR and to inhibit the PDGF-dependent cell proliferation and protection from apoptosis. These results demonstrate that Cbl can negatively regulate PDGFR-dependent biological responses and that this function requires the conserved tyrosine kinase binding domain of Cbl.

Cbl, the 120-kDa cytoplasmic polypeptide product of the c-cbl proto-oncogene, is the cellular homologue of v-cbl, a retroviral oncogene that induces pre-B lymphomas and myeloid leukemias in mice (1). A number of recent studies have established Cbl as a component of the signal transduction cascades downstream of both the receptor protein tyrosine kinases (PTKs) and surface receptors noncovalently associated with PTKs (2–6). Cbl is rapidly and prominently phosphorylated on tyrosine residues upon stimulation through a number of receptors, resulting in the association of Cbl with SH2 domain-containing proteins, such as the p85 subunit of PI 3-kinase, the Crk adapter protein family, and the Rac exchange factor VAV.

Recently, the phosphopeptide motifs that mediate these interactions have been localized within the C-terminal third of Cbl (7–10). Furthermore, a large proline-rich region (amino acids 481–690) in Cbl mediates interactions with the SH3 domains of Src family PTKs and the adaptor proteins Grb2 and Nck, thus promoting the formation of signaling protein complexes that are present in cells prior to receptor activation. Although these associations have promoted the notion that Cbl functions as a complex adapter protein to couple PTKs to downstream signaling pathways, lack of evolutionary conservation of the C-terminal region suggests that the primary role of Cbl may be different. Indeed, a number of recent biochemical and genetic studies have identified Cbl as a novel negative regulator of receptor and non-receptor PTKs (2–6).

The first evidence for the role of Cbl as a negative regulator of tyrosine kinase signaling was provided by studies of vulval development in Caenorhabditis elegans, a process that requires the LET-23 receptor tyrosine kinase, a homologue of the mammalian epidermal growth factor receptor (EGFR) (11). A negative regulator of signaling downstream of the LET-23 receptor, the suppressor of lineage defect 1 (slt-1), was shown to encode a Cbl homologue (SLI-1). Recently, a Drosophila Cbl homologue (D-Cbl) was also identified and shown to function as a negative regulator of the Drosophila EGFR-mediated 7 photoreceptor development (12, 13). Notably, the loss of function mutations in SLI-1, including one missense point mutation (G315E), mapped to the evolutionarily conserved N-terminal region (Cbl-N) which functions as a tyrosine kinase binding (TKB) domain in Cbl (14). The Cbl TKB domain was shown to bind to the ligand of the LET-23 receptor, LET-23, and the ligand of the SLT-1 receptor, S.A.

The abbreviations used are: PTK, protein tyrosine kinase; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; GST, glutathione S-transferase; TKB, tyrosine kinase-binding; HRPO, horseradish peroxidase; PDGFR, platelet-derived growth factor receptor; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; Tyr(P), phosphotyrosine; α-MEM, α-minimal essential medium; FCS, fetal calf serum; HA, hemagglutinin; aa, amino acid(s); TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; PI 3-kinase, phosphatidylinositol 3-kinase.

1 The region referred to as the TKB domain was previously referred to as a phosphotyrosine-binding (PTB) domain, but structural studies show that this region is an integrated phosphopeptide-binding platform composed of a four-helical domain, an EF hand, and an SH2 domain.
the negative regulatory phosphorylation sites within the SH2 kinase linker of ZAP70 and Syk PTks (15–17). These binding activities were abrogated by a mutation (G306E), equivalent to the loss of function mutation (G315E) in SLI-1. Notably, Cbl overexpression in the rat basophilic leukemia cell line RBL-2H3 led to a decrease in the autophosphorylation and kinase activity of Syk and inhibition of degranulation in response to FceRI stimulation (18); the Cbl-induced reduction in Syk tyrosine kinase activity requires an intact Cbl TK domain (16). Overexpression of Cbl in Jurkat T cells was shown to reduce Ras-dependent AP1 transcription factor activity, whereas overexpression of the oncogenic 70Z/3 Cbl mutant induced an enhancement of basal and Ca2+ ionophore-induced nuclear factor of activated T cell reporter activity (9, 19). These studies have highlighted the role of Cbl as a negative regulator of ZAP70/Syk PTks.

A potential role for Cbl as a negative regulator of mammalian receptor PTks is suggested by a number of findings. Decreased autophosphorylation of the EGFR and lower JAK-STAT phosphorylation was observed in NIH3T3 cells that overexpressed Cbl, and higher EGFR autophosphorylation was seen in antisense Cbl-transfected cells (20). Conversely, NIH3T3 cells expressing oncogenic forms of Cbl revealed a hyperphosphorylation of PDGFRα and an up-regulation of signaling downstream of this receptor (21). In addition, NIH3T3 cells expressing the oncogenic forms of Cbl showed an increase in the autophosphorylation and kinase activity of a transfected human EGFR, both under serum-starved and EGF-stimulated conditions (22). Apparently, the effect of the oncogenic mutants of Cbl on PDGFRα and EGFR reflects a reversal of the negative regulatory role of endogenous wild type Cbl.

Although the above studies clearly implicated Cbl as a negative regulator of PTks, the biochemical and cell biological mechanisms mediating such an effect have been less clear. Recently, we showed that Cbl overexpression in NIH3T3 cells led to the enhancement of the PDGF-induced ubiquitination and degradation of the PDGFRα (23). These findings suggested that one mechanism of the negative regulatory function of Cbl for receptor PTks may be at the level of regulating the ligand-induced turnover of PDGFRα. Here, we have extended these initial observations along three major directions. First, we show that Cbl regulates both the α and β PDGFR receptors. Second, we show that an intact TKB domain is required for Cbl-dependent enhancement of ubiquitination and degradation of the PDGFR. Finally, we demonstrate that Cbl-mediated negative regulation of the PDGF receptors is biologically relevant in that Cbl overexpression induces a TKB domain-dependent reduction in PDGF-induced cell proliferation. These studies generalize the role of Cbl in regulating receptor PTk signaling and suggest a direct correlation between the biological functions of Cbl and its ability to regulate ligand-induced receptor ubiquitination and degradation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture**—Parental and transfected NIH3T3 cells were cultured in α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS, HyClone Laboratories, Inc., Logan, UT). Transfectants of the PDGFRα-negative Patch mutant mouse-derived 3T3 cell line (Ph) (28) were cultured in Dulbecco’s modified Eagle’s medium containing 10% FCS. For all transfectants, the medium was supplemented with 500 μg/ml G418 (Life Technologies, Inc.). Transfectants were established by retroviral transfection using pJZenNeo vector-transduced (Ph-pJ.1 and Ph-pJ.2) Ph cell lines were also established using retroviral transfection. The overexpression of exogenous HA-Cbl in NIH3T3 (Fig. 1A) and Ph (Fig. 1C, middle and bottom panels) transfectants was demonstrated by anti-Cbl and anti-HA immunoblotting of whole cell lysates. The expression of PDGF receptors in NIH3T3 transfectants was quantified by anti-PDGFRα and anti-PDGFRβ immunoblotting of serial dilutions of whole cell lysates (Fig. 1B). The expression of PDGF-Rβ in Ph cell transfectants was assessed by anti-PDGFRβ immunoblotting (Fig. 1C, top panel). Whereas different clones varied in the level of PDGFR expression under basal (unstimulated) conditions, no systematic correlation between PDGF levels and the nature of Cbl (wild type versus G306E) or its level of expression was apparent.

**Antibodies**—The murine monoclonal antibodies used were as follows: 4G10 (anti-phosphotyrosine, anti-Tyr(P)) (24) (gift of Dr. Brian Druker, Oregon Health Sciences University) and 12CA5 (anti-HP epo tag) (25). The polyclonal rabbit antibodies used were as follows: anti-PDGFRα (sc-431, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-PDGFRβ (against the kinase insert; PharMingen, San Diego, CA), anti-PDGFRβ (Upstate Biotechnology, Lake Placid, NY) was used for Western blotting in Figs. 1, B and C, and 4C; and anti-ubiquitin (NCL-UBIQ, NovoCastra Laboratories, Newcastle, UK) obtained from Vector Laboratories, Burlingame, CA. Normal rabbit serum (negative control) was obtained from non-immunized rabbits.

**PDGF Stimulation**—For PDGF stimulation, cells were first cultured for 24 h in medium containing 0.5% FCS (serum deprivation). Recombinant human PDGF-AA (for selective activation of PDGFRα) or PDGF-BB (both from Upstate Biotechnology Inc., Lake Placid, NY) was then added at a final concentration of 20 ng/ml. At the indicated time points, the medium was aspirated, and cell extracts were prepared at 4 °C in a lysis buffer containing 0.5% Triton X-100 (Fluka, Buchs, Switzerland), 50 mM Tris, pH 7.5, 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 μg/ml each of leupeptin, pepstatin, chymostatin, antipain, and aprotinin (Sigma).

**Immunoprecipitation and Immunoblotting**—Optimal amounts of antibodies (determined by preliminary titrations) were added to aliquots of lysates equalized for protein content by the Bradford assay (Bio-Rad, using the bovine serum albumin standard). After 1–2 h of rocking at 4 °C, 20 μl of protein A-Sepharose 4B beads (Amersham Pharmacia Biotech) were added, and incubation was continued for 45 min. Beads were washed six times in lysis buffer, and bound proteins were solubilized in sample buffer with 2-mercaptoethanol and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Resolved polypeptides were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilion-P, Millipore, Bedford, MA) and immunoblotted with the indicated antibodies. Horseradish peroxidase (HRPO)-conjugated protein A (Cappel-Organon Technika, Durham, NC) was used as a secondary reagent for blotting, followed by enhanced chemiluminescence (ECL, NEN Life Science Products). Membranes were stripped and reprobed, as described (21). Graphics were generated by direct scanning of films using a Hewlett-Packard ScanJet 4c™ scanner and Corel Draw™ version 6 software.

**In Vitro GST Fusion Protein Binding Experiments**—Six-mg aliquots of cell lysates were incubated with glutathione-Sepharose beads on which GST or GST fusion proteins of Cbl (Cbl-N, aa 1–357; Cbl-N/ G306E; or Cbl-C, aa 358–906) were noncovalently immobilized (14). After 1 h of preincubation at 4°C, cells were washed five times in cold wash buffer (0.1% Triton X-100, 50 mM Tris, pH 8.0, 150 mM NaCl), and proteins were eluted by boiling in Laemmli sample buffer containing 2-mercaptoethanol. Samples were resolved by SDS-PAGE and analyzed by Western blotting.

**Biostin Labeling of Cell-surface PDGFRα and Assessment of Its Ligand-induced Degradation**—Cell monolayers were washed with ice-cold phosphate-buffered saline containing 20 mM HEPES buffer solution, pH 7.35, and then incubated in the same buffer with sulfo-NHS-biotin (Pierce) for 15 min at 4°C. After washing, the cells were incubated in α-MEM containing 1 mg/ml bovine serum albumin (tissue culture grade; Sigma) with PDGF-BB (7.5 ng/ml) at 37°C for the indicated time points, and lysates were prepared as described above. Anti-PDGFRβ immunoprecipitates of these lysates were resolved by SDS-PAGE and blotted with an avridin-HRPO conjugate (Vector Laboratories, Burlingame, CA) followed by enhanced chemiluminescence, as described above. Densitometry was carried out using Scion Images for Windows™ software. Densitometric data are expressed in arbitrary units.
Assessment of PDGF-dependent Cell Proliferation—The cells were plated in triplicate at a density of \(2 \times 10^4\) cells per 25-cm\(^2\) flask in α-MEM containing 0.5% FCS. After 18 h, the cells were switched to the same medium supplemented with 20 ng/ml recombinant human PDGF-AA or PDGF-BB, as appropriate. The culture medium, containing the indicated PDGF isoforms, was changed on alternate days. The number of cells was counted every other day from day 1 to day 13, or as indicated, using a hemacytometer.

TUNEL Assay for Assessment of Apoptosis—The cells were grown on coverslips in α-MEM containing 0.5% FCS and 20 ng/ml PDGF-BB for 10 days, fixed in 4% paraformaldehyde in phosphate-buffered saline, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After a series of washes in phosphate-buffered saline, DNA fragmentation in apoptotic cells was determined by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay using the In Situ Cell Death Detection Kit, POD (Roche Molecular Biochemicals), according to the instructions of the manufacturer. Apoptotic cells were visualized and photographed using a fluorescence microscope. Propidium iodide was used to visualize the entire cell population on the coverslip.

RESULTS

Efficient Tyrosine Phosphorylation of Cbl Requires an Intact TKB Domain—Given that the G306E mutation in the TKB domain of Cbl, corresponding to a loss-of-function mutation in \(C.\) elegans Cbl homologue SLI-1, abrogates the ability of an oncogenic Cbl mutant to transform NIH3T3 cells and to induce hyperphosphorylation of the PDGFR\(\alpha\) (21), it appeared likely that a functional TKB domain would be required for Cbl-mediated negative regulation of the PDGFR\(\alpha\). To assess if this was the case, we established NIH3T3 clones expressing either wild type Cbl (Cbl.8, Cbl.9, and Cbl.2–8, lanes 5–7) or with Cbl-G306E (G306E.1–10 and G306E.2–3, lanes 3 and 4). 100 µg of each lysate was resolved by SDS-PAGE, transferred to a PVDF membrane, and serially immunoblotted with anti-Cbl (top panel) and anti-HA (bottom panel) antibodies. B, 100, 50, or 25 µg of each cell lysate was resolved by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with anti-PDGFR\(\alpha\) (top panel) or anti-PDGFR\(\beta\) (bottom panel) antibodies. C, whole cell lysates were prepared from Ph cells transfected with pJZenNeo vector alone (Ph-pJ.1 and Ph-pJ.2) or with pJZenNeo constructs encoding HA epitope-tagged wild type Cbl (Ph-Cbl.1–8, Ph-Cbl.2–8, and Ph-Cbl.2–10). 100 µg of each lysate was resolved by SDS-PAGE, transferred to a PVDF membrane, and serially immunoblotted with anti-PDGFR\(\beta\) (top panel), anti-Cbl (middle panel), and anti-HA (bottom panel) antibodies.
**Fig. 2.** PDGF-AA-induced tyrosine phosphorylation of wild type Cbl versus Cbl-G306E mutant and their association with the PDGFRα. A, serum-deprived NIH3T3 transfectant lines Cbl-2–8 and G306E.1–10 were either left unstimulated (-) or were stimulated with 20 ng/ml PDGF-AA for 3 min (+), and cell lysates were prepared. 1-mg aliquots of each lysate were subjected to immunoprecipitation (IP) with anti-HA antibody 12CA5. Immunoprecipitated polypeptides were resolved by SDS-PAGE, transferred to PVDF membrane, and subjected to anti-Tyr(P) (4G10) immunoblotting (top and middle panels). The membrane was stripped and reprobed with anti-HA antibody (bottom panel). PDGFRα and Cbl are indicated. B, wild type HA-Cbl (Cbl.8 and Cbl.2–8) and HA-Cbl-G306E (G306E.1–10 and G306E.2–3) expressing NIH3T3 cells were either left unstimulated (lanes 1, 5, 9, and 13) or were stimulated with 20 ng/ml PDGF-AA for the indicated times (min), and cell lysates were prepared. 1-mg aliquots of lysates were then subjected to immunoprecipitation (IP) with anti-HA antibody followed by immunoblotting with the same antibody (bottom panel). The membrane was stripped and reprobed with anti-Tyr(P) antibody 4G10 (top panel).

Interestingly, the Cbl-G306E mutant itself exhibited a reduced level of PDGF-induced tyrosine phosphorylation compared with that of wild type Cbl, even though the cell lines examined expressed similar levels of the Cbl proteins (Fig. 2A, middle and bottom panel). The reduced phosphorylation of Cbl-G306E was not due to changes in the kinetics of phosphorylation, as revealed by a time course experiment (Fig. 2B). These results indicate that the TKB domain is essential for efficient PDGF-induced tyrosine phosphorylation of Cbl.

A Critical Role of Cbl TKB Domain in the Enhancement of Ligand-induced Ubiquitination of PDGFRα—To assess directly the role of the TKB domain in Cbl-dependent enhancement of the PDGFRα ubiquitination, we compared NIH3T3 cells transfected with vector alone (pJF) to cells expressing wild type Cbl (Cbl.2–8) or Cbl-G306E (G306E.1–10 and G306E.2–3). Serum-deprived cells were stimulated with PDGF-AA for various time points, and anti-PDGFRα immunoprecipitations were carried out on their lysates. These were analyzed by anti-Tyr(P) and anti-ubiquitin immunoblotting (Fig. 3, A and B, top and middle panels).

As expected (23), the ligand-induced ubiquitination of the PDGFRα (seen as the upward-shifted smear in anti-Tyr(P) blot) in Cbl-overexpressing NIH3T3 cells was substantially higher as compared with that in the vector-transfected cells and was already maximal at 3 min of PDGF stimulation (Fig. 3A, lane 6) versus about 10 min in the control cells (Fig. 3A, lane 3). Notably, the Cbl-dependent enhancement of PDGFRα ubiquitination was essentially abrogated by the G306E mutation in Cbl (Fig. 3B, compare lanes 2–4 to lanes 6–8 and 10–12). These results reveal a critical role for the TKB domain in Cbl-mediated facilitation of ligand-induced PDGFRα ubiquitination.

Association of Cbl with PDGFRβ and Enhancement of the Ligand-induced Ubiquitination of PDGFRβ by Cbl Overexpression—Elucidation of signaling pathways that are either shared between α and β PDGF receptors, or are selectively recruited to these receptors, is of significant biological interest (27). Our previous studies of NIH3T3 cells expressing the wild type Cbl (23) or the oncogenic Cbl proteins (21) have established the PDGFRα as a target of the negative regulatory effect of Cbl; this effect appears to involve the Cbl-dependent enhancement of the ligand-induced ubiquitination and degradation of the PDGFRα. However, it has remained unclear whether PDGFRα is selectively regulated by Cbl or if Cbl-dependent regulation may be a feature of both the α and β PDGF receptors. To address this question, we overexpressed HA-tagged wild type Cbl in PDGFRα-negative Patch 3T3 cells (Cbl-Phe) (28), using retroviral transfection (Fig. 1C). Similar to parental Ph cells, the Cbl-Phe cells failed to respond to PDGF-AA by tyrosine phosphorylation of cellular proteins (data not shown).

Stimulation of Cbl-Phe cells with PDGF-BB was used to assess if selective stimulation of the PDGFRβ can induce tyrosine phosphorylation of Cbl and its association with the activated receptor. For this purpose, lysates prepared from unstimulated or PDGF-BB-stimulated Cbl-Phe cells were subjected to anti-HA immunoprecipitation and analyzed by anti-Tyr(P) immunoblotting.

Stimulation of Ph-Cbl cells with PDGF-BB induced a prominent time-dependent phosphorylation of Cbl, which peaked at 3 min (Fig. 4A). When anti-HA and anti-PDGFRβ immunoprecipitates were resolved side-by-side, the 185-kDa Cbl-associated band (Fig. 4B, lane 4) co-migrated with PDGFRβ. In addition, the 185-kDa band was immunodepleted with anti-PDGFRβ antibody (data not shown). These experiments indicated that, similar to selective stimulation of the PDGFRα, selective stimulation of the PDGFRβ can also induce Cbl phosphorylation and its association with the receptor.

Our previous studies showed that Cbl could physically interact with the PDGFRα through the Cbl TKB domain, which binds directly to autophosphorylated PDGFRα (21). In addition, the C-terminal region of Cbl can associate with the acti-
vated receptor, consistent with its ability to bind the SH3 domains of Src family kinases and adaptor proteins, such as Grb2, which interact with autophosphorylated PDGFRα (29–31). To assess if Cbl could bind to PDGFRβ via similar mechanisms, in vitro binding assays were performed using GST fusion proteins that incorporated the TKB domain of Src family kinases and adaptor proteins, such as Grb2, which interact with autophosphorylated PDGFRα for the indicated time points (min), and cell lysates were prepared. 1-mg aliquots of each lysate were used for immunoprecipitation with anti-PDGFRα antibody. The immunoprecipitated polypeptides were resolved by SDS-PAGE, transferred to PVDF membrane, and probed with anti-Tyr(P) and anti-ubiquitin antibodies (top panels). A and B represent separate experiments.

PDGF receptors.

To determine if Cbl overexpression affects ligand-induced ubiquitination of the PDGFRα, serum-deprived parental NIH3T3 cells or wild type Cbl-transfected NIH3T3 cells (Cbl.8) were stimulated with PDGF-BB for the indicated time points, and anti-PDGFRα immunoprecipitates were analyzed by anti-Tyr(P) and anti-ubiquitin immunoblotting. This experiment revealed that the PDGFRα ubiquitination was substantially enhanced and peaked earlier (3 versus 10 min) in Cbl-overexpressing cells compared with parental cells (Fig. 5A, top and middle panels). Enhancement of the ligand-induced ubiquitination of the PDGFRα was also observed using two additional wild type Cbl-expressing clones (Cbl.2–8) but not in the vector-transfected clone (pJ.8) of NIH3T3 cells (Fig. 5B, top panel and middle panels, compare lanes 2, 6, and 10). Notably, similar to the ubiquitination of PDGFRα, the G306E mutation abrogated the effect of Cbl on the ubiquitination of the PDGFRα (Fig. 5B, top and middle panels, lanes 13–20). These results demonstrate that, similar to PDGFRα, the PDGFRβ is also a target of Cbl-dependent enhancement of ligand-induced ubiquitination and that a functional Cbl TKB domain is re-

![Fig. 4. Association of Cbl with PDGFRβ and enhancement of ligand-induced PDGFRβ ubiquitination in Cbl-overexpressing NIH3T3 cells. A and B, PDGFR-BB-dependent phosphorylation of Cbl and its association with PDGFRβ. HA-tagged Cbl-overexpressing Patch cells were serum-deprived and either left unstimulated (−) or were stimulated with 20 ng/ml PDGFR-BB for 3 min (B) or the indicated time points (min) (A), and cell lysates were prepared. 1-mg aliquots of lysates were used for immunoprecipitation with normal rabbit serum (NRS, control), anti-HA antibody, or anti-PDGFRβ antibody. Immunoprecipitated polypeptides were resolved by SDS-PAGE, transferred to PVDF membrane, and subjected to immunoblotting with anti-Tyr(P). A, the membrane was first immunoblotted with anti-HA antibody (bottom panel). B, binding of PDGFRβ to GST fusion proteins of Cbl. PDGFRβ-negative Patch cells were either left unstimulated (−) or were stimulated with 20 ng/ml recombinant human PDGFR-BB for 10 min (+) prior to lysis. Binding reactions were carried out by incubating glutathione-Sepharose beads coated with 10 μg of GST, GST-Chl-N (Cbl aa 1–357), GST-Chl-C (Cbl aa 358–906), or GST-Chl-N-G306E with 6-mg aliquots of cell lysates for 4 h. Bound polypeptides were resolved by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with anti-PDGFRβ antibody (bottom panel). The membrane was stripped and reprobed with anti-Tyr(P) antibody (top panel).]
Cbl Enhances the Ligand-dependent Degradation of PDGFRβ in a TKB Domain-dependent Manner—Given our previous findings that overexpression of Cbl facilitated ligand-dependent degradation of the PDGFRα (23), we tested the effect of Cbl overexpression on the turnover of the PDGFRβ.

For this purpose, we quantified the PDGFRβ levels in vector-transfected NIH3T3 cells and cells overexpressing either wild type Cbl (Cbl.8, Cbl.9, Cbl.2–8) or Cbl-G306E mutant (G306E.1–10, G306E.2–3) were either left unstimulated (−) or were stimulated with 20 ng/ml PDGF-BB for the indicated times (min), and cell lysates were prepared. 1-mg aliquots of each lysate were subjected to immunoprecipitation with anti-PDGFRβ antibody, resolved by SDS-PAGE, transferred to PVDF membrane, and subjected to anti-PDGFRβ blotting (bottom panel). The membranes were stripped and serially reprobed with anti-ubiquitin (middle panel) and anti-Tyr(P) (top panel) antibodies. A and B represent separate experiments.

As shown in Fig. 6, a time-dependent decrease in the intensity of the receptor band was observed following ligand stimulation of all cell lines. However, whereas a significant level of biotinylated PDGFRβ was still detectable at 90 min after PDGF stimulation in vector-transfected NIH3T3 cells and cells overexpressing Cbl-G306E mutant (G306E.1–10, G306E.2–3), relatively little PDGFRβ were detected in Cbl-transfected cells even at the 60-min time point (Fig. 6A, lane 3).

The loss of cell-surface PDGFRβ in these cells was quantified by densitometry of the streptavidin blot (Fig. 6B). This analysis highlights a substantially earlier and more pronounced loss of surface-labeled PDGFRβ upon PDGF stimulation of the Cbl-overexpressing NIH3T3 cells as compared with that in the vector-transfected NIH3T3 cells. The half-life of the surface-labeled PDGFRβ following PDGF-BB stimulation was 41–52% shorter in Cbl-transfected NIH3T3 cells compared with that in parental cells (Cbl.9, 21 min and Cbl.2–8, 26 min versus pJ.1, 44 min). Notably, the G306E mutation abrogated the effect of Cbl on the degradation of the PDGFRβ; the half-life of the surface-labeled PDGFRβ of Cbl-G306E transfectants was close to that detected in vector-transfected NIH3T3 cells (G306E.1–10, 32 min; G306E.2–3, 39 min). Similar results were observed in two additional experiments (data not shown). Together, these results show that the enhancement of PDGFRβ ubiquitination as a result of Cbl overexpression is associated with a faster ligand-induced loss of the surface PDGFRβ and that a functional TKB domain is essential for this effect.

Cbl Inhibits PDGF-dependent Cell Proliferation in a TKB Domain-dependent Manner—Given that Cbl overexpression led to enhanced ubiquitination and degradation of both the α and β PDGF receptors, and the essential role of the evolutionarily conserved TKB domain in this process, we wished to ascertain if the regulatory influence of Cbl led to any biological consequences. For example, a faster down-regulation of the activated PDGFRs could be expected to decrease the mitogenic effects of PDGF. To test if this was indeed the case, we com-
pared the PDGF-induced proliferation of vector-transfected NIH3T3 cells with cells overexpressing either the wild type Cbl or its TKB domain mutant (Cbl-G306E).

All cell lines showed relatively little proliferation when grown in low serum medium (0.5% FCS) (Fig. 7A, top left panel). To quantify PDGF-dependent proliferation, cells were cultured in the low serum medium containing 20 ng/ml PDGF-AA and were counted every other day. The Cbl-overexpressing clones showed a decrease in PDGF-AA-dependent growth, with about half as many cells harvested at each time point beyond day 3, when compared with the vector-transfected NIH3T3 cells (Fig. 7A, top right panel). The retarded growth was also observed in two additional Cbl-overexpressing clones (data not shown). Importantly, the Cbl-G306E-transfected cells showed little or no retardation of growth in PDGF-AA (Fig. 7A, top right panel). Cbl-transfected NIH3T3 cells also showed a reduction of proliferative response to PDGF-AA, as compared with the vector-transfected cells; at early time points (days 1–5), the rate of proliferation of Cbl-G306E-expressing cells in response to PDGF-AA was similar to that of vector-transfected cells, but a substantial reduction was noted at later time points (days 9–13) (Fig. 7A, bottom left panel).

The above results were contradictory to our earlier studies (21); we did observe reduced proliferation in a Cbl-transfected clone of NIH3T3 cells (Cbl.8) compared with parental NIH3T3 cells. Since our earlier studies had been carried out using a late passage of Cbl.8, we reanalyzed an early passage of this clone that was comparable to other clones analyzed above. Indeed, a reduction in PDGF-BB-induced proliferation was also observed using this early passage of the Cbl.8 clone (Fig. 7A, bottom right panel). Furthermore, a late passage of this clone, comparable to that used in our earlier studies (21), did not show reduced proliferative response to PDGF-AA (data not shown). Apparently, long term culture selects for cells that have compensated for the Cbl-induced decrease in PDGF responsiveness.

Since NIH3T3 cells express both PDGFRα and -β, and a β-selective ligand does not exist, we could not selectively stimulate PDGFRβ in these cells. Therefore, to confirm further the effect of the overexpression of Cbl on PDGFRβ-dependent cell proliferation, we compared the PDGF-induced proliferation of vector-transfected Ph cells with that of Ph cells overexpressing the wild type Cbl. All clonal cell lines expressed comparable levels of PDGFRβ (Fig. 1C, top panel). All of the cell lines showed relatively little proliferation when grown in low serum medium (0.1% FCS) (Fig. 7B, left panel). When cells were cultured in the low serum medium containing 20 ng/ml PDGF-BB (Fig. 7B, right panel).
PDGF-BB and counted every other day, the Cbl-overexpressing Ph clones showed lower PDGF-BB-dependent growth at each time point beyond day 3, when compared with the vector-transfected Ph clones (Fig. 7, middle and bottom panels). The data points represent the mean of triplicates; error bars show ± 1 S.D.

The viability of NIH3T3 transfectants cultured with PDGF, as measured by trypan blue staining, was more than 95% until day 7 (Fig. 8, middle and bottom panels, and data not shown), indicating that the effect of Cbl was primarily mediated through reduced proliferation rather than increased cell death. However, a higher proportion of Cbl-transfected NIH3T3 cells, as compared with parental or vector-transfected NIH3T3 cells, underwent death at later time points. In the experiment shown in Fig. 7, the viability of Cbl transfectants decreased to about 95% by day 9, whereas the viability of control cells at the same time point was about 95% (Fig. 8). When NIH3T3 transfectants were cultured in low serum (0.5% FCS) medium, the Cbl-transfected cells showed a higher degree of cell death compared with control cells at day 9 (Fig. 8, top panel). All cell lines showed a decrease in cell viability at later time points (day 11 and day 13). These results suggest that, in addition to reduced proliferation, wild type Cbl overexpression also enhances cell death in transfected cells at later time points.

Morphological examination of Cbl-overexpressing cells at day 9 (when reduced viability was clearly observed) revealed a larger proportion of cells with shrunken cytoplasm and condensed nuclei, whereas the vast majority of vector-transfected cells grew as monolayers of flat refractile cells (data not shown). These observations suggested that the observed cell death may be due to apoptosis. A TUNEL assay was performed to assess if this was the case.

Compared with relatively few TUNEL-positive cells in the cultures of vector-transfected and Cbl-G306E-transfected cells (Fig. 9, A and E), a larger proportion of TUNEL-positive cells was observed in cultures of Cbl-transfected cells. The apoptotic cells are clearly visible with their nuclear fragmentation (Fig. 9C, see arrows). In addition, several dead cells are visible as condensed bright spots apparently reflecting late stage apoptosis (Fig. 9C). Propidium iodide staining demonstrated that lack of TUNEL-positive cells in vector-transfected or Cbl-G306E-transfected cells was not due to lower cell density on the coverslips (Fig. 9, B, D, and F). Overall, these results indicate that Cbl overexpression induces a TKB domain-dependent reduction of PDGF-induced cellular proliferation and that Cbl-overexpressing cells are sensitized to undergo apoptotic cell death.

**DISCUSSION**

Recent studies have clearly identified the Cbl proto-oncoprotein as a negative regulator of receptor and non-receptor tyrosine kinases. However, the precise biological consequences of this negative regulation, such as alterations in proliferation, differentiation, or metabolic activity of a cell, have not been defined. Here, we provide evidence that Cbl-dependent negative regulation of the PDGF receptors provides a mechanism to down-regulate the cellular proliferation initiated by PDGF. We also demonstrate that Cbl physically interacts with and functionally regulates PDGFRβ, similar to our earlier observations with the PDGFRα, thus suggesting a general role for Cbl as a negative regulator of PDGF receptors. Finally, we demonstrate that the N-terminal TKB domain of Cbl, which is highly conserved through evolution and is the site of loss-of-function mutations in *C. elegans* Cbl homologue SLI-1, is essential for a functional effect of Cbl on PDGF receptors.

A role for Cbl to regulate negatively the cellular proliferation in response to growth factors has been implied previously but has not been experimentally demonstrated. In fact, previous studies, including our own analysis of one Cbl-transfected NIH3T3 clone (Cbl.8), failed to detect a difference in the rate of proliferation between cells overexpressing Cbl and the parental NIH3T3 cells (20, 21, 32). These earlier findings had assessed both serum-dependent and PDGF-induced proliferation. We confirmed the lack of an effect of Cbl overexpression on the proliferation of the late-passage Cbl.8 clone used in our earlier studies (data not shown). To clarify this discrepancy, we derived an additional clone (Cbl.9) from the same bulk transfec tant that was used to derive Cbl.8, and in addition we derived an independent Cbl-overexpressing NIH3T3 clone Cbl.2–8. These clones were examined relatively early after cloning (typically 10–30 passages). These analyses revealed a substantial and reproducible reduction in the PDGF-AA and PDGF-BB-induced proliferation of Cbl-transfected NIH3T3 cells compared with those transfected with the vector alone. A similar reduction in PDGF-induced proliferation was also observed using an early passage of the Cbl.8 clone (Fig. 7A). Apparently, the growth disadvantage conferred by Cbl overexpression is selected against, and continuous culture appears to promote growth of cells that have compensated for the inhibitory effect of Cbl on proliferation.

We have also observed a reduction in serum- and EGF-dependent proliferation of the Cbl-overexpressing NIH3T3 cells (data not shown), indicating that Cbl-mediated negative regulation of PTKs indeed impinges on the proliferative outcome of receptor activation. This conclusion is consistent with the increased serum or PDGF-induced proliferation of NIH3T3 cells transformed with oncogenic Cbl mutants (21). Our results support the likelihood that increased hypercellularity in various organs such as thymus, spleen, and mammary gland of Cbl−/−
mice (33, 34) may derive, at least in part, from the loss of the negative regulatory effect of Cbl on cellular proliferation triggered by extracellular growth factors.

Our earlier finding of a general up-regulation of the PDGFRα signaling cascade in the oncogenic Cbl-transfected NIH3T3 cells (21) suggested that the effect of Cbl on PDGF-induced cellular proliferation is likely to involve a general reduction in the level of various PDGFR-initiated signaling pathways. Extracellular growth factors, including PDGF, are involved in the regulation of both mitogenesis and cell survival (27) Thus, an overall reduction of signals emanating from the PDGFR could coordinately reduce mitogenesis and cell survival in Cbl-overexpressing cells. PDGF-dependent reduction in proliferation in Cbl-overexpressing cells was detectable at relatively early time points (e.g. 3–5 days) when no detectable cell death was observed (Fig. 7A). However, clear evidence of reduced cell survival was seen at later time points (after 7–9 days). These data are consistent with an overall reduction of PDGFR-mediated signals upon Cbl overexpression. However, it remains possible that Cbl may selectively influence certain signaling pathways. In particular, the tendency of Cbl-overexpressing cells to undergo cell death is notable. Distinct signaling pathways have been implicated in the regulation of cell survival and the induction of mitogenesis in response to certain growth factors (35). Notably, the PI 3-kinase pathway is thought to provide a key mechanism to promote cell survival (36). Given the association of Cbl with the p85 subunit of PI 3-kinase, via Cbl phosphotyrosine 731 (9, 10), it remains possible that Cbl may sequester PI 3-kinase and reduce the efficiency of a signaling pathway that ensures cell survival. However, more detailed mutational and biochemical analyses will be required to precisely delineate the basis of reduced proliferation and enhanced apoptosis observed upon Cbl overexpression.

The present study provides direct data to show that PDGFRβ is a target of the negative regulatory function of Cbl. This result is significant for a number of reasons. Our previous studies showed that the expression of oncogenic Cbl proteins in NIH3T3 cells led to an up-regulation of the PDGFRα signaling cascade, but limited analyses of the PDGFRβ revealed no obvious alterations in its signaling cascade. These results raised the possibility that Cbl may preferentially affect the PDGFRα signaling (21). An independent study reported that PDGF-BB stimulation of NIH3T3 cells led to Cbl phosphorylation, but Cbl-PDGFRβ association was not detected (37). PDGF-BB-induced phosphorylation of Cbl and its association with PDGFRβ in Patch cells (Fig. 4, A and B), which lack the expression of PDGFRα, directly establishes that Cbl participates in signaling via the PDGFRβ. The reasons for the earlier observation of apparently selective deregulation of PDGFRα signaling in oncogenic Cbl-transfected NIH3T3 cells are not clear. It is possible that the availability of PDGFRα ligand in the serum and/or the relative levels of PDGFRα versus PDGFRβ at the time of the analysis were contributing factors. It is also likely that the failure to detect the PDGFRβ-Cbl association in a previous investigation (37) reflects a combination of the relatively low level of the endogenous Cbl in NIH3T3 cells and the inefficiency of the available antibodies in immunoprecipitating PDGFRβ (data not shown).

Cbl overexpression also led to an enhancement of ligand-induced ubiquitination and degradation of the PDGFRβ. Given the correlation of enhanced receptor ubiquitination with receptor degradation (38), Cbl is therefore likely to provide a mechanism of negative regulation for the PDGFRβ similar to its role for the PDGFRα. Consistent with this suggestion, mutations in the cytoplasmic tail of the PDGFRβ that reduced its ligand-induced ubiquitination also led to reduced ligand-dependent degradation and a higher mitogenic response to PDGF (39). Furthermore, a substantial reduction in PDGF-BB-induced cell proliferation was observed in NIH3T3 cells overexpressing Cbl, similar to their reduced response to PDGF-AA (Fig. 7A), and was also observed in PDGFRα-negative Ph cells overexpressing Cbl. Overall, these results strongly suggest that Cbl regulates...
both the PDGFRα and PDGFRβ.

Recent studies have revealed the crucial importance of the N-terminal tyrosine kinase-binding domain of Cbl for its negative regulatory influence on PTKs. This domain is highly conserved during evolution (1, 11–13) and is the site of loss-of-function mutations in the C. elegans Cbl homologue SLI-1 (11). By itself, this region is oncogenic and up-regulates PDGFRα signaling, whereas a mutation (G306E) corresponding to a loss-of-function mutation in SLI-1 abrogated both activities. In addition, Cbl phosphorylation was still capable of physical association with the PDGFR as well as on PDGFR ubiquitination and degradation. In addition, Cbl phosphorylation was dramatically reduced when the TKB domain was mutated. Importantly, however, Cbl-G306E was still capable of physical association with the PDGFRs, likely via Src family kinases, Grb2, or other unidentified adaptor molecules. Thus, the binding of the TKB domain of Cbl to activated receptor PTKs appears to be critical for a functional effect rather than merely a mechanism for stable association with PDGFR receptors. Notably, a reduced EGF-induced phosphorylation of Cbl-G306E with the retention of its physical association with the EGFR has also been observed (26). In contrast, we have observed that an intact TKB domain is essential for the physical association of Cbl with the cytoplasmic PTK Syk, as well as for the Cbl-dependent negative regulation of Syk (16).

In conclusion, studies reported in this paper generalize the negative regulatory role of Cbl for the PDGFR family and demonstrate that the tyrosine kinase binding domain of Cbl is indispensable for the functional effects of Cbl. Importantly, our studies demonstrate that Cbl-mediated biochemical effects on the PDGFR lead to important biological consequences, as shown by a reduction in cellular proliferation in responses to PDGFR ligands. Further mechanistic studies of Cbl-mediated negative regulation of the PDGFR signaling cascade are likely to elucidate a novel mode of biological control of cellular responses to extracellular stimuli.

Acknowledgments—We thank Dr. Daniel F. Bowen-Pope for providing the Patch cell line and for reading the manuscript. We thank members of the Band laboratory for helpful suggestions and Navin Rao for critical reading of the manuscript.

REFERENCES
1. Blake, T. J., Shapiro, M., Morse, H. C. D., and Langdon, W. Y. (1991) Oncogene 6, 653–657
2. Miyake, S., Lupher, M. L., Jr., Andoniou, C. E., Lill, N. L., Ota, S., Douillard, P., Rao, N., and Band, H. (1997) Crit. Rev. Oncog. 8, 189–218
3. Lupher, M. L., Jr., Andoniou, C. E., Bonita, D., Miyake, S., and Band, H. (1998) Int. J. Biochem. Cell Biol. 30, 439–444
4. Langdon, W. Y. (1995) Aust. N. Z. J. Med. 25, 859–864
5. Smit, L., and Borst, J. (1997) Crit. Rev. Oncog. 8, 359–379
6. Liu, Y. C., and Altman, A. (1998) Cell. Signalling 10, 377–385
7. Andoniou, C. E., Thiern, C. B., and Langdon, W. Y. (1996) Oncogene 12, 1981–1989
8. Marengere, L. E., Mirtissis, C., Kozierebucki, I., Veillette, A., Mak, T. W., and Penninger, J. M. (1997) J. Immunol. 159, 70–76
9. Liu, Y. C., Elly, C., Langdon, W. Y., and Altman, A. (1997) J. Biol. Chem. 272, 168–173
10. Feshchenko, E. A., Langdon, W. Y., and Tsygankov, A. Y. (1998) J. Biol. Chem. 273, 8325–8331
11. Yoon, C. H., Lee, J., Jongeward, G. D., and Sternberg, P. W. (1995) Science 269, 1102–1105
12. Meisner, H., Daga, A., Buxton, J., Fernandez, B., Chawla, A., Banerjee, U., and Czec, M. P. (1997) Mol. Cell. Biol. 17, 2217–2225
13. Hime, G. R., Dhungat, M. P., Ng, A., and Bowtell, D. D. (1997) Oncogene 14, 2709–2719
14. Lupher, M. L., Jr., Reidquist, K. A., Miyake, S., Langdon, W. Y., and Band, H. (1996) J. Biol. Chem. 271, 24063–24068
15. Lupher, M. L., Jr., Sungyang, Z., Shoelson, S. E., Cantley, L. C., and Band, H. (1997) J. Biol. Chem. 272, 33140–33144
16. Lupher, M. L., Jr., Rao, N., Lill, N. L., Andoniou, C. E., Miyake, S., Clark, E. A., Druker, B., and Band, H. (1998) J. Biol. Chem. 273, 35273–35281
17. Deckert, M., Elly, C., Altman, A., and Liu, Y. C. (1998) J. Biol. Chem. 273, 8867–8874
18. Ota, Y., and Samelson, L. E. (1997) Science 276, 418–420
19. Bellothan, B. L., Graham, I. J., Stoica, B., Debell, K. E., and Bonvini, E. (1997) J. Biol. Chem. 272, 30806–30811
20. Ueno, H., Sasaki, K., Miyagawa, K., Honda, H., Mitani, K., Yazaki, Y., and Hirai, H. (1997) J. Biol. Chem. 272, 8739–8743
21. Bonita, D. P., Miyake, S., Lupher, M. L., Jr., Langdon, W. Y., and Band, H. (1997) Mol. Cell. Biol. 17, 4597–4610
22. Thien, C. B., and Langdon, W. Y. (1997) Oncogene 15, 2909–2919
23. Miyake, S., Lupher, M. L., Jr., Druker, B., and Band, H. (1998) Proc. Natl. Acad. Sci. U. S. A. 85, 7927–7932
24. Druker, B. J., Mann, H. M., and Roberts, T. M. (1989) N. Engl. J. Med. 321, 1383–1391
25. Wilson, I. A., Niman, H. L., Houghten, R. A., Chenreson, A. R., Connolly, M. L., and Lerner, R. A. (1984) Cell 37, 767–772
26. Thien, C. B., and Langdon, W. Y. (1997) Oncogene 14, 2239–2249
27. Claesson-Welsh, L. (1994) Prog. Growth Factor Res. 5, 57–54
28. Seifert, R. A., van Koppen, A., and Bowen-Pope, D. F. (1993) J. Biol. Chem. 268, 4473–4480
29. Gelderblom, J. A., Rosenkrantz, S., Bazenet, C., and Kazlauskas, A. (1998) J. Biol. Chem. 273, 5908–5915
30. Hooishmand-Rad, R., Yokote, K., Heldin, C. H., and Claesson-Welsh, L. (1998) J. Cell Sci. 111, 607–614
31. Bazenet, C. E., Gelderblom, J. A., and Kazlauskas, A. (1996) Mol. Cell. Biol. 16, 6926–6936
32. Ojaniemi, M., Langdon, W. Y., and Vuori, K. (1998) Oncogene 16, 3159–3167
33. Murphy, M. A., Schnall, R. G., Venter, D. J., Barnett, L., Bertoncello, I., Thiern, C. B., Langdon, W. Y., and Bowtell, D. D. (1998) Mol. Cell. Biol. 18, 4872–4882
34. Naramura, M., Kole, H. K., Hu, R. J., and Gu, H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15547–15552
35. Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) J. Biol. Chem. 272, 24063–24068
36. Walker, F., Kato, A., Goeze, J. I., Hibbs, M. L., Pouliot, N., Levitzki, A., and Burgess, A. W. (1998) Mol. Cell. Biol. 18, 7192–7204
37. Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. E., and Greenberg, M. E. (1997) Science 275, 661–665
38. Galiest, M. L., Dikic, I., Batzer, A. G., Langdon, W. Y., and Schlessinger, J. (1995) J. Biol. Chem. 270, 20242–20245
39. Mori, S., Heldin, C. H., and Claesson-Welsh, L. (1992) J. Biol. Chem. 267, 6429–6434
40. Mori, S., Heldin, C. H., and Claesson-Welsh, L. (1993) J. Biol. Chem. 268, 577–583
41. Meng, W., Sawadikosol, S., Burakoff, S. J., and Eck, M. J. (1999) Nature 398, 84–90