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Phosphotyrosine Binding Domain-Dependent Upregulation of the Platelet-Derived Growth Factor Receptor α Signaling Cascade by Transforming Mutants of Cbl: Implications for Cbl's Function and Oncogenicity

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Recent studies have demonstrated that Cbl, the 120-kDa protein product of the c-*cbl* proto-oncogene, serves as a substrate of a number of receptor-coupled tyrosine kinases and forms complexes with SH3 and SH2 domain-containing proteins, pointing to its role in signal transduction. Based on genetic evidence that the *Caenorhabditis elegans* Cbl homolog, SLI-1, functions as a negative regulator of the LET-23 receptor tyrosine kinase and our demonstration that Cbl's evolutionarily conserved N-terminal transforming region (Cbl-N; residues 1 to 357) harbors a phosphotyrosine binding (PTB) domain that binds to activated ZAP-70 tyrosine kinase, we examined the possibility that oncogenic Cbl mutants may activate mitogenic signaling by deregulating cellular tyrosine kinase machinery. Here, we show that expression of Cbl-N and two other transforming Cbl mutants (CblY368Δ and Cbl366-382Δ or Cbl70Z), but not wild-type Cbl, in NIH 3T3 fibroblasts leads to enhancement of endogenous tyrosine kinase signaling. We identified platelet-derived growth factor receptor α (PDGFRα) as one target of mutant Cbl-induced deregulation. In mutant Cbl transfectants, PDGFRα was hyperphosphorylated and constitutively complexed with a number of SH2 domain-containing proteins. PDGFRα hyperphosphorylation and enhanced proliferation of mutant Cbl-transfected NIH 3T3 cells were drastically reduced upon serum starvation, and PDGF-AA substituted for the maintenance of these traits. PDGF-AA stimulation of serum-starved Cbl transfectants induced the in vivo association of transfected Cbl proteins with PDGFRα. In vitro, Cbl-N directly bound to PDGFRα derived from PDGF-AA-stimulated cells but not to that from unstimulated cells, and this binding was abrogated by a point mutation (G306E) corresponding to a loss-of-function mutation in SLI-1. The Cbl-N/G306E mutant protein, which failed to induce enhanced growth and transformation of NIH 3T3 cells, also failed to induce hyperphosphorylation of PDGFRα. Altogether, these findings identify a novel mechanism of Cbl's physiological function and oncogenesis, involving its PTB domain-dependent direct interaction with cellular tyrosine kinases.

The binding of extracellular growth factors to their cognate cell surface receptors triggers a cascade of biochemical events that culminates in cell proliferation. Orderly execution of these biochemical events and integration of multiple biochemical signaling pathways ensure normal cellular proliferation. Alterations in the components of these signaling pathways can lead to loss of regulatory control, resulting in uncontrolled cell division and, ultimately, oncogenic transformation. Indeed, recent biochemical and genetic studies have revealed that a number of proteins involved in mitogenic growth factor signaling are encoded by genes originally identified as proto-oncogenes. Studies designed to examine these oncogenic proteins have not only provided insight into how they can render cells tumorigenic but have also shed much light on the function of their normal cellular counterparts (1, 4, 11). One such protein that has recently come under scrutiny is the product of the proto-oncogene c-*cbl*, first identified as the cellular homolog of v-*cbl*, the transforming gene of the murine retrovirus Cas-NS-1 (27). v-*cbl*, which represents a fusion of the first 355 amino-terminal residues of the full-length (913 amino acids [aa]) protein to viral *gag* sequences, induces pre-B-cell lymphomas and myelogenous leukemias in neonatally inoculated mice (7, 27).

Although the oncogenicity of v-Cbl in mice and its ability to acutely transform immortal rodent fibroblasts in vitro have been well documented, the function of wild-type Cbl, which is nontransforming even when overexpressed (2), has remained unclear. Only the C-terminal region, which is absent from the v-Cbl oncoprotein, contains primary sequences predicted to form functional motifs or domains (Fig. 1). These include a proline-rich region (aa 481 to 690, 23% proline; Fig. 1, PRO) which provides potential binding motifs for Src homology 3 (SH3) domain-containing proteins (7, 34); a leucine zipper (aa 857 to 892; Fig. 1, LZ) which, by analogy to other proteins with this motif, could mediate intermolecular oligomerization (7, 26); and a Cys_3-His-Cys_4 ring finger (aa 380 to 425; Fig. 1, RF) structurally related to other zinc-coordinating domains and thought to mediate potential protein-protein or protein-DNA interactions (41). Interestingly, a mutant form of Cbl with a 17-aa deletion (aa 366 to 382; Fig. 1, 70Z/3Δ) immediately amino terminal to the ring finger and C terminal to the v-Cbl coding region was cloned from a pre-B lymphoma cell line (70Z/3) and found to be oncogenic when transfected into NIH

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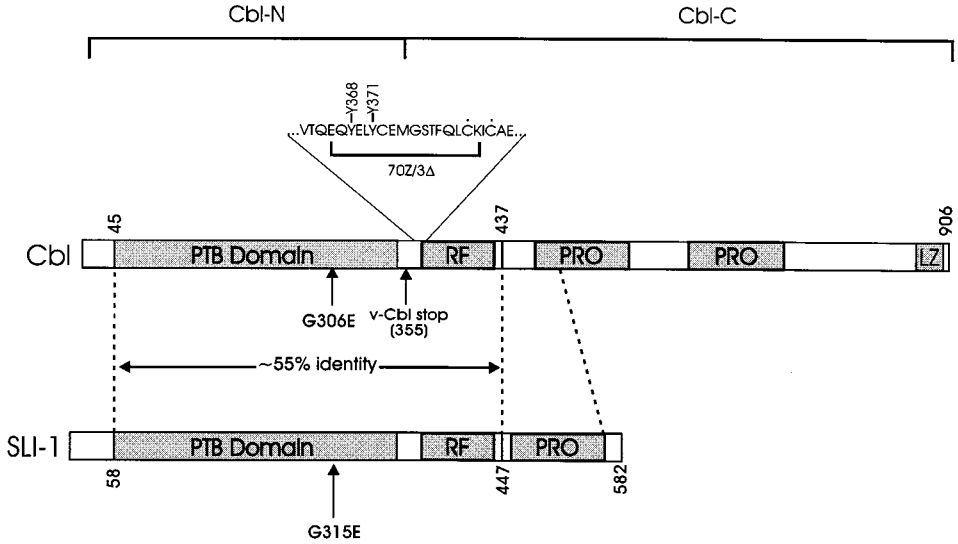

FIG. 1. Schematic of functional domains within Cbl and its comparison to SLI-1, its *C. elegans* homolog. The N-terminal transforming region (aa 1 to 355 [murine] or 1 to 357 [human]; Cbl-N) corresponds to sequences present in the viral Cbl (v-Cbl) oncoprotein. The remaining C-terminal region (Cbl-C) contains sequences predicted to form the following functional domains: a Cys_3-His-Cys_4 ring finger domain (aa 381 to 425; RF), proline-rich regions (aa 481 to 690, 23% proline; PRO) with multiple potential binding motifs for SH3 domain-containing proteins, and a leucine zipper (aa 857 to 892; LZ). The positions of oncogenic point deletion mutations (Y368 and Y371) and a naturally occurring 17-aa deletion (70Z/3Δ) are indicated. In contrast to the 906-aa human Cbl polypeptide, SLI-1 is only 582 aa long. However, residues 58 to 447 of SLI-1 (corresponding to Cbl-N and the ring finger) share 55% identity with human Cbl residues 45 to 437. The tentative position of the PTB domain within Cbl-N and the locations of loss-of-function mutations (G306E in Cbl and G315E in SLI-1; vertical arrows) are also indicated. The most C-terminal proline-rich region in SLI-1 exhibits high similarity to the most N-terminal proline-rich region in human Cbl (dashed diagonal line). Dots above cysteine residues denote the start of the ring finger domain.

3T3 fibroblasts (2, 6). Additionally, mutant proteins with small deletions within this 17-aa stretch or a point deletion of the tyrosine at position 368 or 371 (Fig. 1, Y368 and Y371), but not tyrosine-to-phenylalanine substitutions, also induced tumorigenic transformation of NIH 3T3 cells (2). These analyses suggest a role of the ring finger region in negatively regulating Cbl's transforming, and perhaps its normal biochemical, function(s).

Insights into the biochemical basis of Cbl's physiological role and transforming potential have come from several lines of recent investigation. A number of oncogenic mutant forms of Cbl incorporating deletions near the ring finger (above) were found to be constitutively tyrosine phosphorylated in pre-B leukemia cells (70Z/3 mutant) or when expressed in NIH 3T3 cells (2, 41). Furthermore, normal Cbl was found to be constitutively hyperphosphorylated on tyrosine in cells expressing oncogenically activated Abl (v-Abl or BCR/Abl), Src, or Lck tyrosine kinase (2, 36, 43). In a complementary line of investigation, Cbl was demonstrated to be an early and dominant substrate of tyrosine phosphorylation in response to activation of cell surface receptors coupled to tyrosine kinases. These include the cell surface receptors coupled to nonreceptor tyrosine kinases, such as the T- and B-cell antigen receptors (13, 14, 19, 24, 33, 36), the granulocyte-macrophage colony-stimulating factor receptor, the interleukin 3 and erythropoietin receptors (3, 32), the Fcγ receptor (43), the TPO/c-mp1 receptor (40), and the CD38 receptor (25), as well as the growth factor receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) (8, 17, 20, 30, 43), the platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor 1 (FGFR-1), nerve growth factor receptor (20), and the CSF-1 receptor (43, 46). Furthermore, receptor activation was shown to induce complexes of tyrosine-phosphorylated Cbl with the SH2 domains of the p85 subunit of the phosphatidylinositol 3 (PI3)-kinase and Crk adaptor proteins (9, 14, 19, 21, 24, 29, 32, 33, 37, 38). Cbl was also demonstrated to form activation-independent complexes with the SH3 domains of Nck and GRB2 adapter proteins, and the GRB2-associated fraction of Cbl was a prominent substrate of tyrosine phosphorylation in both hematopoietic and nonhematopoietic cells (9, 14, 19, 29, 32, 33, 39).

Taken together, these findings have provided strong support for Cbl's participation in signaling downstream of a diverse array of receptors coupled to tyrosine kinases. These data also suggested that the hyperphosphorylation on tyrosine observed on several oncogenic mutant Cbl proteins (above) may provide a biochemical mechanism for Cbl's transforming ability by promoting constitutive complexes of Cbl and SH2 domain-containing signaling proteins, such as Crk and PI3-kinase p85, which are involved in the regulation of mitogenic signaling. Surprisingly, however, the N-terminal transforming region of Cbl (aa 1 to 357; equivalent to Cbl-derived sequences in v-Cbl and referred to as Cbl-N in this report) lacks the SH3 domain-binding proline-rich region and the demonstrated or predicted SH2 domain binding sites and does not show detectable tyrosine phosphorylation (7, 8, 28), suggesting a distinct mechanism of its oncogenic activity and potential physiological function. The comparison of the primary structure of Cbl-N with known signaling proteins, however, did not reveal any predictable functional domain(s) (5).

The possibility that Cbl-N harbors a functional domain(s) relevant to both normal signal transduction and tumorigenesis has been suggested by recent genetic and biochemical studies. Analyses of the vulval developmental pathway in *Caenorhabditis elegans* identified a gene, suppressor of lineage defect 1 (*sli-1*), as a negative regulator of the signaling downstream of the LET-23 receptor tyrosine kinase, a homolog of the mammalian EGFR (23). The sequence of human Cbl including Cbl-N and its ring finger domain is 55% identical to the corresponding regions in SLI-1 (Fig. 1), indicating strong evolu-

tionary conservation (48). A point mutation (G315E) in SLI-1 that induces a loss of its function resides within this region (Fig. 1) (23). Taken together with the dominant oncogenic potential of Cbl-N, these findings strongly suggested the presence of a functional domain(s) within the N-terminal region of Cbl. Indeed, we have recently demonstrated that Cbl-N harbors a phosphotyrosine-binding (PTB) domain that selectively binds to ZAP-70 tyrosine kinase in T lymphocytes (Fig. 1) (28). Importantly, the ability of the Cbl-N PTB domain to bind to ZAP-70 was abrogated by a point mutation (G306E) corresponding to the *C. elegans* SLI-1 loss-of-function mutation G315E (Fig. 1). Altogether, these analyses suggest that PTB domain-mediated interaction with cellular tyrosine kinases may be an integral feature of Cbl-induced oncogenesis and its normal physiological function.

To elucidate the mechanism of Cbl-induced transformation and to shed light on the role of Cbl's PTB domain in the oncogenic process, we have undertaken an analysis of NIH 3T3 cells transfected with wild-type and oncogenic forms of Cbl. We demonstrate that oncogenic Cbl mutants upregulate endogenous tyrosine kinase signaling machinery. We identify PDGFRα as one of the tyrosine kinases and provide evidence for its interaction with Cbl-N. This interaction is abrogated by a G306E mutation which inactivates its PTB domain. These results, together with genetic studies with *C. elegans* and our studies with T cells, identify a novel mechanism of Cbl's physiological function and oncogenesis, through a PTB domain-dependent regulation of cellular tyrosine kinases.

MATERIALS AND METHODS

Cell lines and culture. Murine NIH 3T3 fibroblasts were maintained in α-minimal essential medium (α-MEM) supplemented with 10 mM HEPES (pH 7.4), 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100-U/ml penicillin, 100-μg/ml streptomycin (GIBCO-BRL), and 10% fetal calf serum (FCS; Hyclone) (complete medium). Cells were released with trypsin (0.05%)-EDTA (0.5 mM) and subcultured at a 1:10 to 1:20 ratio every 3 to 5 days.

Human Cbl cDNA constructs in the pJZenNeo retroviral vector encoding Cbl proteins with a hemagglutinin (HA) epitope tag (between residues 2 and 3) have been previously described (2, 22). These include wild-type Cbl (Cbl-wt), a mutant Cbl with aa 366 to 382 deleted (Cbl-70Z), and a mutant Cbl with a deletion of residue 368 (Cbl-368Δ). These expression plasmids were transiently transfected into the PA317 murine retrovirus packaging cell line by calcium phosphate-mediated transfection (Stratagene transfection kit). After 48 h, overnight supernatants were collected and used to infect NIH 3T3 cells for 3 h in the presence of Polybrene (4 μg/ml). Transfectants were selected in 0.5-mg/ml G418 (GIBCO-BRL) and seeded at limiting dilution. Isolated colonies were picked by using cloning cylinders, expanded, and analyzed for HA-tagged Cbl expression as described below. The Cbl-N transfected NIH 3T3 cell line used in the experiments whose results are described in Fig. 2 to 9 was derived by calcium phosphate-mediated transfection (Stratagene) with plasmid pSRαNeo.HACbl-N, which encodes Cbl residues 1 to 357 tagged with an HA epitope between residues 2 and 3. This expression plasmid was previously derived by subcloning an HA–Cbl-N cDNA insert from pJZenNeo into vector pSRαNeo (10). The HA–Cbl-N and HA–Cbl-N/G306E transfectants used in the experiment whose results are shown in Fig. 11 were generated by retroviral infection by using cDNA constructs in vector pJZenNeo as described previously (2). HA–Cbl-N- and HA–Cbl-N/G306E-transfected cells were used as pooled populations.

Serum deprivation and PDGF stimulation. For serum deprivation, cells were plated overnight in 100-mm-diameter petri dishes in complete α-MEM with 10% FCS. Cells were then washed once with phosphate-buffered saline (PBS) and incubated in α-MEM containing 0.5% FCS for the times indicated in Results. For the stimulation experiments, cells were serum deprived as described above for 48 h and then transferred to α-MEM containing 0.5% FCS supplemented with 20-ng/ml recombinant human PDGF-AA (Upstate Biotechnology Inc. catalog no. 01-309). At the time points indicated in Results, medium was aspirated and cells were lysed by adding cold lysis buffer (see below).

Cell proliferation assay. Cells were plated in quadruplicate at a density of 5×10^3 cells (see Fig. 8A) in medium with 0.5% FCS per 25-cm^2 flask. After 24 h, cells were switched to either the same medium, medium with 10% FCS, or medium with 0.5% FCS supplemented with 20-ng/ml PDGF-AA. Respective media were added fresh on day 4. On day 7, cells were released with trypsin-EDTA and counted in a hemocytometer. As a control, untransfected NIH 3T3 cells and wild-type Cbl-expressing cells (Cbl-wt.8) were plated in triplicate at a density of 2.5×10^4/25-cm^2 flask in α-MEM containing 0.5% FCS. After 24 h, cells were treated as described above except that medium was added fresh on day 3 and cells were released and counted on day 5 (see Fig. 8B).

Antibodies. The monoclonal antibodies used were 4G10 [antiphosphotyrosine; αTyr(P)] (15), anti-phospholipase Cγ1 (αPLC-γ1; Upstate Biotechnology Inc. catalog no. 05-163), 12CA5 (anti-HA epitope tag; αHA) (47), OKT8 (αCD8; American Type Culture Collection), and anti-glutathione *S*-transferase (anti-GST; 42). The polyclonal rabbit antibodies used were anti-PDGFRα (αPDGFRα; Santa Cruz Biotechnology catalog no. sc-431), anti-PDGFRβ (αPDGFRβ; Santa Cruz Biotechnology catalog no. sc-432), anti-GRB2 (αGRB2; Santa Cruz Biotechnology catalog no. sc-255), anti-Cbl (αCbl; Santa Cruz Biotechnology catalog no. sc-170), anti-PI3-kinase p85 subunit (αp85; Transduction Laboratories catalog no. P13030), and anti-SHC (αSHC; Transduction Laboratories catalog no. S14630). Normal rabbit serum (NRS) was obtained from unimmunized rabbits. Horseradish peroxidase (HRPO)-conjugated protein A or sheep anti-mouse immunoglobulin (Cappel-Organon Technika) was used as the second-step reagent for immunoblotting.

Immunoprecipitations. Cells were lysed by scraping with a rubber policeman in ice-cold lysis buffer containing 0.5% Triton X-100 (Fluka), 150 mM sodium chloride, 50 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, and 1 mM sodium orthovanadate (Sigma Chemical Co.). Insoluble material was removed by centrifugation at $10{,}000 \times g$ for 20 min. Antibodies were added to aliquots of precleared lysates equalized for protein by the Bradford assay (BSA standard; BioRad) and Coomassie staining. After 1 to 2 h of rocking at 4°C, a 20-μl volume of protein A-Sepharose 4B beads (Pharmacia) was added and incubation was continued for 45 min. The beads were washed five times in cold lysis buffer, and immune complexes were eluted by boiling in Laemmli sample buffer containing 5% 2-mercaptoethanol (2-ME). Samples were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore) for Western blotting.

In vitro GST fusion protein binding experiments. Precleared lysates were incubated overnight with GST, or GST fusion proteins made with Cbl-N (aa 1 to 357) or Cbl-N/G306E noncovalently immobilized on glutathione-Sepharose beads (28). The beads were washed six times in cold lysis buffer, and proteins were eluted by boiling in Laemmli sample buffer with 2-ME. Samples were resolved by SDS-PAGE and analyzed by Western blotting.

Western blotting. Following the transfer of proteins, PVDF membranes were blocked at room temperature (RT) with 2% gelatin in Tris-buffered saline–Tween (TBS-T; 10 mM Tris-HCl [pH 8], 150 mM NaCl, 0.05% Tween 20) with 0.02% sodium azide for 2 h to overnight. Membranes were then incubated with an optimal concentration of the primary antibody in TBS-T–azide for 1 to 2 h at RT, washed six times in TBS-T, and incubated with HRPO-conjugated protein A (1:20,000 dilution in TBS-T) for 45 min. Membranes were washed six times in TBS-T and visualized by enhanced chemiluminescence (ECL) with autoradiographic film in accordance with the manufacturer's recommendations (NEN Life Sciences). For reprobing, membranes were stripped in 62.5 mM Tris-HCl (pH 6.7)–2% SDS–0.1 M 2-ME for 30 min at 50°C, rinsed twice in TBS-T, and blocked with 2% gelatin in TBS-T–azide prior to primary antibody incubation.

Filter-binding assay. Anti-Tyr(P) or anti-PDGFRα immunoprecipitates were prepared from precleared cell lysates, resolved by SDS-PAGE, and transferred to PVDF membranes. Membranes were blocked in 2% gelatin–TBS-T–azide at RT and then probed with 2.5 μg of eluted GST fusion proteins per ml. After six washes in TBS-T, filters were serially probed by incubation with an anti-GST monoclonal antibody and a sheep anti-mouse–HRPO conjugate and blots were detected by ECL (18, 28). Membranes were stripped and reprobed with an anti-Tyr(P) antibody.

RESULTS

Previous findings that certain oncogenic Cbl mutants are constitutively phosphorylated on tyrosine and that phosphorylation of Cbl upon stimulation of tyrosine kinase-coupled cell surface receptors leads to its association with SH2 domain-containing proteins (2, 3, 9, 13, 14, 19–21, 24, 25, 29, 32, 33, 36–38, 40, 43, 46) suggested that mutant Cbl-induced oncogenesis may proceed through enhanced recruitment of downstream SH2 domain-containing signaling proteins. However, the Cbl sequences retained in the v-Cbl protein (Cbl-N) lack the predicted SH2 domain-binding motifs and the SH3 domain-binding proline-rich region and, when expressed in fibroblasts or T cells, do not show detectable constitutive tyrosine phosphorylation. These findings suggested an alternative mechanism of oncogenesis shared between Cbl-N and other oncogenic mutant forms of Cbl. In view of our recent finding that Cbl-N harbors a PTB domain that binds selectively to the tyrosine kinase ZAP-70 in T cells, we considered the possibility

that oncogenesis by Cbl mutant proteins may proceed through PTB domain-dependent activation of a cell's endogenous tyrosine kinase machinery. To test this notion, we established NIH 3T3 fibroblast transfectants expressing either wild-type human Cbl (Cbl-wt) or the three mutant forms Cbl-368Δ, Cbl-70Z [Cbl366-382Δ], and Cbl-N (Cbl aa 1 to 357) (2). All of the introduced proteins were tagged near the N terminus (between aa 2 and 3) with an HA epitope to allow detection with anti-HA antibody 12CA5 (47). As observed previously (2), NIH 3T3 transfectants expressing Cbl mutant proteins exhibited a transformed morphology whereas the wild-type Cbl-expressing cells remained untransformed (see Fig. 11A) (data not shown). Furthermore, mutant Cbl transfectants exhibited a three- to fourfold higher cell number at saturation density and produced colonies when seeded in soft agar whereas the wild-type Cbl transfectants failed to grow in soft agar (data not shown). These transfectants were subjected to biochemical analyses of tyrosine kinase-mediated signaling as described below.

Increased tyrosine phosphorylation of endogenous cellular polypeptides in NIH 3T3 cells expressing oncogenic Cbl mutants. We reasoned that if the expression of oncogenic Cbl mutants induces deregulation of the endogenous tyrosine kinase machinery, then we could expect an alteration in the phosphotyrosine content of the endogenous polypeptides, in addition to previously observed hyperphosphorylation of introduced mutant Cbl proteins (2). To assess if this was the case, anti-HA or anti-Tyr(P) immunoprecipitations were done with lysates of NIH 3T3 transfectants cultured in complete culture medium, resolved by SDS–7.5% PAGE, transferred to PVDF membrane, and immunoblotted with anti-Tyr(P) antibody 4G10. Anti-CD8 immunoprecipitates served as a negative control (Fig. 2).

Initial analysis of multiple NIH 3T3 transfectant clones (indicated by numeric extensions of cell line designations) expressing wild-type Cbl (Cbl-wt.8 and Cbl-wt.14) or oncogenic mutant forms of Cbl (Cbl-368Δ.1, Cbl-368Δ.6, Cbl-368Δ.17, Cbl-70Z.15, and Cbl-70Z.18) revealed that mutant Cbl proteins were prominently tyrosine phosphorylated whereas wild-type Cbl was not (Fig. 2A, αHA immunoprecipitates, lanes 2, 5, 8, 11, 14, 17, and 20). In keeping with the increased tyrosine phosphorylation of the mutant Cbl proteins, anti-Tyr(P) antibody immunoprecipitated a substantial amount of these phosphoproteins (Fig. 2A, αCbl blot, lanes 9, 12, 15, 18, and 21) whereas it did not immunoprecipitate detectable amounts of Cbl in Cbl-wt transfectants (Fig. 2A, lanes 3 and 6). Similar results were obtained by anti-HA immunoblotting of anti-Tyr(P) immunoprecipitations (data not shown). These data confirmed our earlier findings made with independently derived NIH 3T3 transfectants (2).

Significantly, anti-Tyr(P) immunoblotting of anti-Tyr(P) immunoprecipitates revealed that a number of additional cellular polypeptides, distinct from the introduced Cbl proteins (apparent mobilities of 185, 125, 87, 75, 60, and 52 kDa; arrows in Fig. 2A) were tyrosine phosphorylated to a greater extent in the mutant Cbl transfectants than in wild-type Cbl transfectants (Fig. 2A, lanes 3, 6, 9, 12, 15, 18, and B, lanes 3 and 4). A similar pattern was observed in anti-Tyr(P) immunoblots of whole cell lysates (Fig. 2C, compare lanes 2, 3, and 4 with lane 1). A polypeptide of 185 kDa (p185) was particularly notable, as its size excluded the possibility that it was a cleavage product of Cbl, and its hyperphosphorylation was the most consistent feature of cells transfected with Cbl-N (Fig. 2B and C, lanes 4). Analysis of Cbl-N transfectants was further revealing, as the transfected Cbl protein (which migrates as an ~40-kDa protein) lacked detectable tyrosine phosphorylation (Fig. 2B, compare lanes 2 and 4). Although the levels of transfected Cbl proteins are not identical among the various transfectants, the increased levels of tyrosine phosphorylation of cellular proteins in mutant Cbl transfectants were not due to higher expression of the mutant Cbl proteins (e.g., compare Cbl-70Z.18 [lane 20] with Cbl-wt.8 [lane 2]). Altogether, these analyses revealed that the expression of oncogenic forms of Cbl resulted in an increase in the phosphotyrosine content of cellular substrates distinct from introduced Cbl proteins, indicating an upregulation of the endogenous tyrosine kinase machinery.

Comigration of hyperphosphorylated p185 with PDGFRα. In view of the enhanced proliferative ability conferred by oncogenic Cbl mutants on NIH 3T3 cells, it appeared likely that the tyrosine kinases which are deregulated by Cbl mutants might include one or more growth factor receptor tyrosine kinases. This possibility was further suggested by the previous findings that a number of growth factors, such as EGF, PDGF, FGF, and nerve growth factor, induce rapid tyrosine phosphorylation of Cbl in NIH 3T3 and other cells (8, 17, 20, 30, 43). Since many of the growth factor receptors known to be expressed on the surface of fibroblasts, such as the PDGFR, the EGFR, and certain FGFRs, migrate in the 170- to 190-kDa range in SDS-PAGE gels, we focused our attention on the p185 protein that was selectively hyperphosphorylated in NIH 3T3 transfectants expressing oncogenic Cbl mutants compared to wild-type Cbl.

First, we assessed if p185 comigrates in SDS-PAGE with any of the growth factor receptors expressed on NIH 3T3 cells. For this purpose, lysates of cells cultured in complete growth medium were subjected to immunoprecipitation with anti-Tyr(P) or anti-growth factor receptor antibodies, followed by anti-Tyr(P) immunoblotting. As seen in Fig. 3, the 185-kDa band is prominently visible in anti-Tyr(P) immunoprecipitates from the two mutant transfectants (Cbl-368Δ.6 and Cbl-70Z.15; lanes 6 and 10), as expected. When using commercially available antibodies against the murine EGFR and FGFRs (FGFR-3 and FGFR-4) for immunoprecipitations, we did not observe a phosphotyrosyl polypeptide comigrating with p185 (data not shown). Significantly, anti-PDGFRα immunoprecipitates revealed a tyrosine-phosphorylated 185-kDa band that was substantially hyperphosphorylated in the mutant Cbl tranfectants compared to the wild-type Cbl transfectant (Fig. 3, compare lanes 7 and 11 with lane 3). Reprobing with anti-PDGFRα antibody demonstrated that this band represented PDGFRα itself (data not shown). Importantly, this band precisely comigrated with the phosphorylated 185-kDa band seen in anti-Tyr(P) immunoprecipitates (Fig. 3, compare lane 6 with lane 7 and lane 10 with lane 11). These comigration analyses strongly suggested that p185 represents PDGFRα. Anti-PDGFRβ immunoprecipitates also showed the presence of a hyperphosphorylated 185-kDa band comigrating with p185, although at a lower intensity than that seen in anti-PDGFRα immunoprecipitates (Fig. 3, compare lane 7 with lane 8 and lane 11 with lane 12). However, immunoblotting with anti-PDGFRβ antibody showed that PDGFRβ migrated faster than p185 (data not shown). Therefore, the presence of p185 in anti-PDGFRβ immunoprecipitates reflects either cross-reactivity of the anti-PDGFRβ antibody with PDGFRα or the formation of PDGFRα/β heterodimers known to be induced by ligand binding (12).

Complete immunodepletion of p185 by antibodies against PDGFRα. While the hyperphosphorylation of PDGFRα in oncogenic Cbl-expressing NIH 3T3 cells and its precise comigration with hyperphosphorylated p185 observed in anti-Tyr(P) immunoprecipitates strongly indicated the identity of p185 with PDGFRα, our attempts to immunoblot p185 [in anti-Tyr(P) immunoprecipitates] with available anti-PDGFRα an-

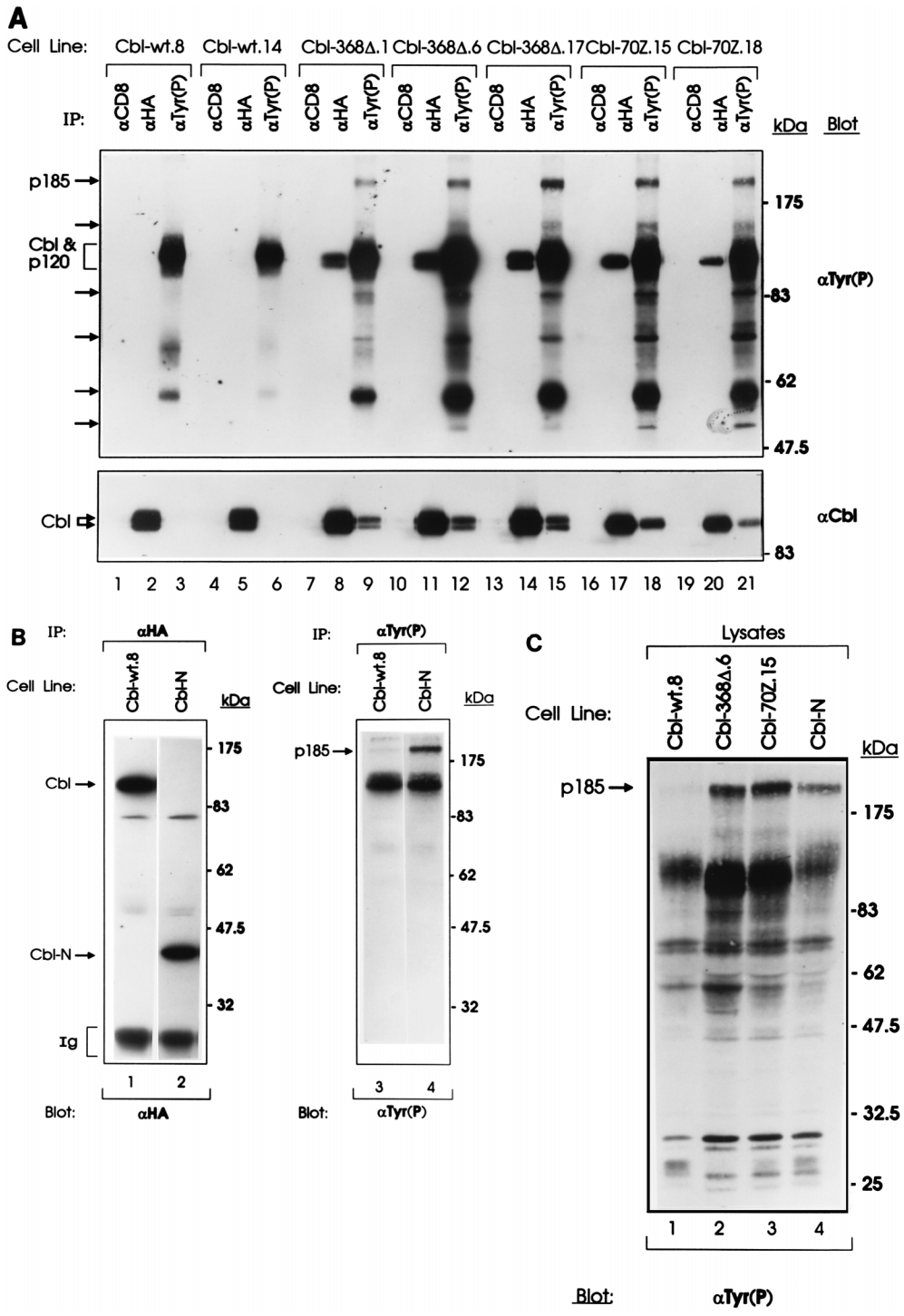

FIG. 2. Expression of oncogenic Cbl proteins in NIH 3T3 fibroblasts results in increased tyrosine phosphorylation of a number of endogenous cellular polypeptides. NIH 3T3 cells expressing HA epitope-tagged Cbl proteins were established by transfection as described in Materials and Methods. Cell line designations: wild-type Cbl-expressing clones, Cbl-wt.8 and Cbl-wt.14; clones expressing the mutant Cbl protein with a tyrosine 368 deletion, Cbl-368Δ.1, Cbl-368Δ.6, and Cbl-368Δ.17; clones expressing mutant Cbl with a 17-aa deletion (aa 366 to 382) corresponding to that isolated from the pre-B leukemia cell line 70Z/3, Cbl-70Z.15 and Cbl-70Z.18; a bulk cell line expressing Cbl-N (aa 1 to 357), corresponding to the region of Cbl found in v-Cbl, Cbl-N. Cell lines were plated in complete culture medium containing 10% FCS for 2 days. Triton X-100 lysates of these cells were used for immunoprecipitation (IP) with OKT8 (anti-CD8) 12CA5 (-HA epitope tag), (or 4G10 [α-Tyr(P)]; (A and B), or for direct analysis (C). For immunoprecipitations, 300 μg of lysate was used whereas 100 μg of lysate was used per lane in panel C. Immunoprecipitated proteins or lysates were separated by SDS-PAGE, transferred to a PVDF membrane, immunoblotted with anti-Tyr(P) antibody followed by a protein A-HRPO conjugate, and detected by ECL. The membrane was stripped and reprobed with anti-Cbl antibody (A). (B) Two identical panels were immunoblotted with anti-HA and anti-Tyr(P) antibodies. Locations of polypeptides exhibiting increased tyrosine phosphorylation are indicated by arrows on the left. Size markers are indicated on right. HA-tagged Cbl (Cbl), Cbl-N, and p185 bands are specifically labeled. An unidentified, constitutively phosphorylated, 120-kDa protein comigrated with Cbl in anti-Tyr(P) immunoprecipitates. Ig, immunoglobulin heavy chain.

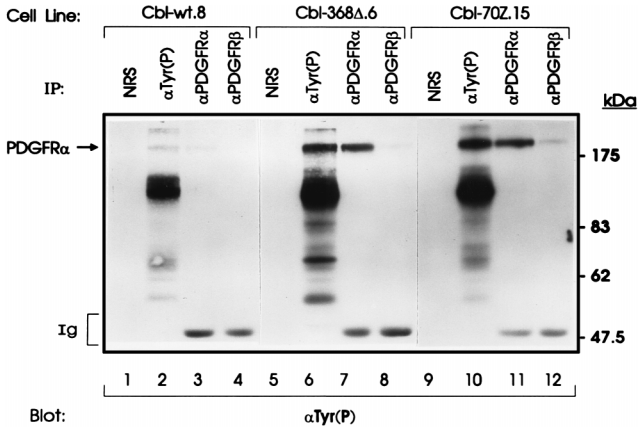

FIG. 3. Hyperphosphorylated p185 in mutant Cbl-transfected NIH 3T3 cells comigrates with PDGFRα. Cbl-wt.8, Cbl-368Δ.6, and Cbl-70Z.15 cells were grown in complete culture medium containing 10% FCS for 2 days. Triton X-100 lysates of these cells were immunoprecipitated with the indicated antibodies (IP), separated by SDS-PAGE, and transferred to a PVDF membrane. The membrane was probed with anti-Tyr(P) antibody, followed by a protein A-HRPO conjugate, and detected by ECL. Ig, heavy chain of immunoglobulin. Note that p185 in anti-Tyr(P) immunoprecipitates precisely comigrated with PDGFRα (arrow on left). Size markers are on the right.

tibodies were not definitive (data not shown). This appeared to be due to a low stoichiometry of phosphorylated versus unphosphorylated PDGFRα under our cell culture conditions and the inefficient immunoblotting of phosphorylated PDGFRα by the available anti-PDGFRα antibodies (data not shown). Therefore, we used immunodepletion analysis to more clearly determine if PDGFRα accounts for part or all of the phosphorylated 185-kDa protein seen in anti-Tyr(P) immunoprecipitates. Cell lysates of two mutant Cbl transfectants (Cbl-368Δ.6 and Cbl-N) were serially precleared three times with either NRS (control) or anti-PDGFRα antibody. Anti-PDGFRα antibody mmunoprecipitation-immunoblot analysis of these lysates showed specific and essentially complete immunodepletion of PDGFRα only in anti-PDGFRα-precleared lysates (Fig. 4A, compare lane 1 with lane 2 and lane 3 with lane 4). These precleared lysates were subjected to immunoprecipitation with anti-Tyr(P) or anti-PDGFRα antibody, followed by anti-Tyr(P) immunoblotting (Fig. 4B).

As expected, anti-Tyr(P) immunoprecipitates of NRS-precleared cell lysates showed a readily detectable p185 that comigrated with phosphorylated PDGFRα (Fig. 4B, compare lane 1 with lane 2 and lane 5 with lane 6). In contrast, p185 was undetectable in the anti-Tyr(P) and anti-PDGFRα immunoprecipitates of PDGFRα-precleared lysates (Fig. 4B, compare lanes 1 and 2 with lanes 3 and 4 and lanes 5 and 6 with lanes 7 and 8). Anti-PDGFRα preclearing selectively depleted p185, as other phosphoproteins were still observed in anti-Tyr(P) immunoprecipitates. These results demonstrate that essentially all of the hyperphosphorylated p185 observed in anti-Tyr(P) immunoprecipitates of mutant Cbl tranfectants is accounted for by hyperphosphorylated PDGFRα. Together, the comigration and immunodepletion analyses clearly establish the identity of hyperphosphorylated p185 in mutant Cbl-transfected NIH 3T3 cells as PDGFRα.

Enhanced association of PDGFRα with SH2 domain-containing signaling proteins in oncogenic Cbl transfectants. Recent analyses have demonstrated that mitogenic signaling through growth factor receptor tyrosine kinases requires recruitment of SH2 domain-containing proteins, such as the p85 subunit of PI3-kinase, PLCγ1, and the adapter proteins GRB2 and SHC, to autophosphorylated receptors (12, 44). Thus, hyperphosphorylation of PDGFRα in mutant Cbl-transfected cells as described above could lead to enhanced recruitment of one or more of the SH2 domain-containing proteins with consequent enhancement of mitogenic signaling. Therefore, we assessed the level of association between PDGFRα and four SH2 domain-containing proteins, namely, GRB2, SHC, PLCγ1, and the p85 subunit of PI3-kinase, by using immunoprecipita-

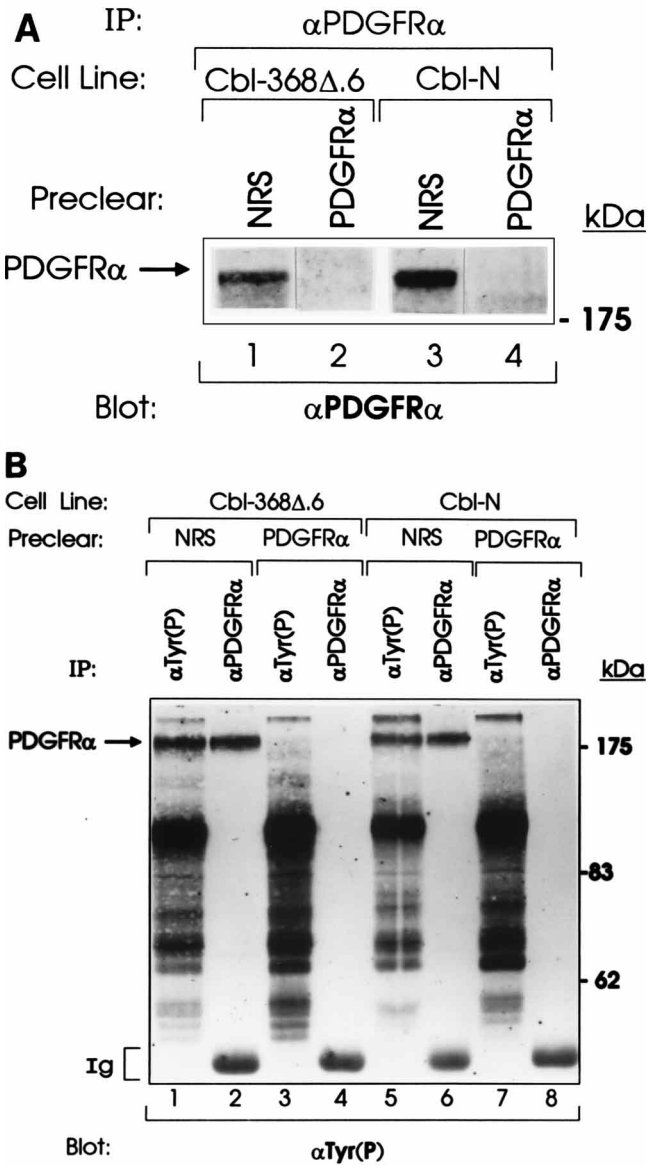

FIG. 4. Complete immunodepletion of hyperphosphorylated p185 in mutant Cbl-transfected NIH 3T3 cells with anti-PDGFRα antibody. NIH 3T3 cells expressing mutant versions of Cbl (Cbl-368Δ.6 and Cbl-N) were plated in complete medium containing 10% FCS for 2 days. Triton X-100 lysates from these cell lines were subjected to three serial immunodepletions with either NRS (as a control) or anti-PDGFRα antibody. The efficiency of immunodepletion was assessed by anti-PDGFRα immunoprecipitation followed by immunoblotting with the same antibody (A). The precleared lysates (1 mg per immunoprecipitation) were then used for immunoprecipitation with anti-Tyr(P) or anti-PDGFRα antibodies followed by anti-Tyr(P) immunoblotting (B). The immunoblot was visualized with a protein A-HRPO conjugate and ECL detection. PDGFRα is indicated on the left. Size markers are on the right. Ig, heavy chain of immunoglobulin.

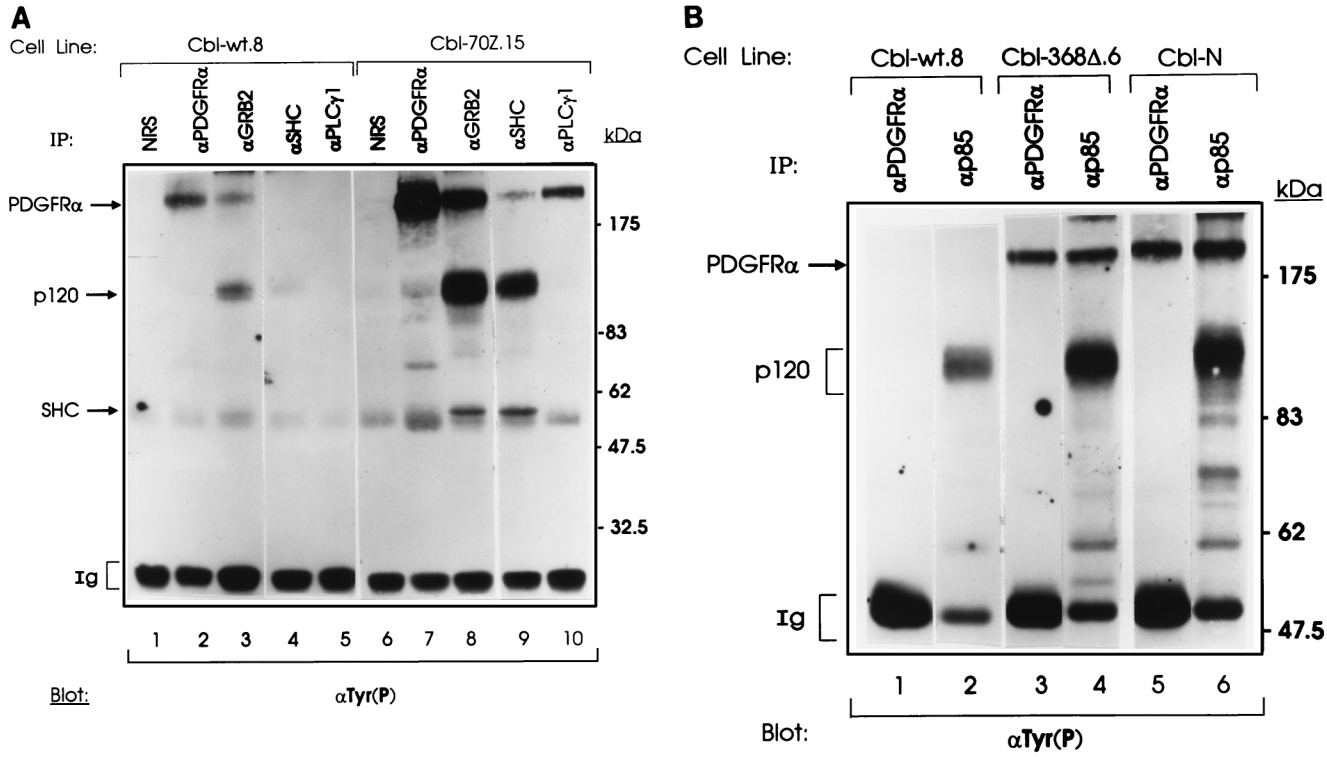

FIG. 5. Increased association of SH2 domain-containing signaling proteins with PDGFRα in oncogenic mutant Cbl proteins. NIH 3T3 cells expressing wild-type (Cbl-wt.8), 70Z mutant (Cbl-70Z.15), Cbl-368Δ mutant (Cbl-368Δ.6), or Cbl-N mutant proteins were cultured in complete culture medium containing 10% FCS for 2 days. Immunoprecipitations (IP) were done with 1-mg samples of Triton X-100 lysates of these cells and NRS (as a control), anti-PDGFRα, anti-Grb2, anti-SHC, and anti-PLCγ1 antibody (A) or anti-p85 (p85 subunit of PI3-kinase) antibody (B). Immunoprecipitated proteins were separated by SDS-PAGE, transferred to a PVDF membrane, probed with anti-Tyr(P) antibody, and detected by using a protein A-HRPO conjugate followed by ECL. A and B represent separate experiments. Various immunoprecipitated species are indicated by arrows on the left. Size markers are on the right. Ig, heavy chain of immunoglobulin.

tion with specific antibodies followed by anti-Tyr(P) immunoblotting.

As seen in Fig. 5A and B, relatively low phosphotyrosine signals were observed in immunoprecipitates of the four SH2 domain-containing proteins from lysates of wild-type Cbl-transfected NIH 3T3 cells (Cbl-wt.8) grown under normal culture conditions (Fig. 5A, lanes 2 to 5, and B, lanes 1 and 2). In contrast, similar immunoprecipitates from transfectants expressing mutant Cbl proteins (Cbl-368Δ.6, Cbl-70Z.15, and Cbl-N) resulted in substantial coimmunoprecipitation of hyperphosphorylated p185 (PDGFRα) (Fig. 5A, lanes 8 to 10, and B, lanes 4 and 6). In addition, a hyperphosphorylated 52-kDa polypeptide, identified as p52 SHC by immunoblotting (data not shown), was observed in anti-GRB2 and anti-SHC immunoprecipitates of mutant Cbl-transfected cells (Fig. 5A, compare lanes 3 and 4 with lanes 8 and 9). Lastly, a substantial increase in the coimmunoprecipitation of an unidentified 120-kDa phosphotyrosyl polypeptide was observed in anti-GRB2, anti-SHC, and anti-p85 immunoprecipitates of mutant Cbl transfectants compared to wild-type Cbl transfectants (p120 in Fig. 5A, compare lanes 3 and 4 with lanes 8 and 9, and in Fig. 5B, compare lane 2 with lanes 4 and 6). This polypeptide, which is constitutively phosphorylated in wild-type Cbl transfectants and hyperphosphorylated in mutant Cbl transfectants, is distinct from Cbl (Fig. 2A and unpublished data). Altogether, these findings demonstrate that expression of mutant Cbl proteins leads to enhanced recruitment of the components of downstream mitogenic signaling machinery to hyperphosphorylated PDGFRα.

Hyperphosphorylation of PDGFRα in mutant Cbl transfectants is reduced upon serum starvation. Hyperphosphorylation of PDGFRα in mutant Cbl-expressing cells and consequent enhanced recruitment of signaling proteins could reflect constitutive activation of PDGFRα or increased responsiveness to its ligand(s). As one test to assess if PDGFRα was constitutively active and as a first step toward assessing responses to authentic PDGFRα ligand (see the next section), we performed serum deprivation studies. To this end, Cbl-wt.8 or Cbl-368Δ.17 cells were plated overnight in complete culture medium and then shifted to medium containing 0.5% FCS (low-serum medium). Lysates were prepared at various time points up to 44 h, and anti-PDGFRα immunoprecipitations of these lysates were analyzed by anti-Tyr(P) and anti-PDGFRα immunoblotting (Fig. 6).

Anti-Tyr(P) immunoblotting revealed a time-dependent decrease in the phosphotyrosine signal on PDGFRα upon serum deprivation of both wild-type and mutant Cbl transfectants. In the wild-type Cbl transfectant, initially low PDGFRα phosphorylation decreased to virtually undetectable levels by 44 h [Fig. 6, αTyr(P) blot, lanes 1 to 6]. While significant tyrosine phosphorylation of PDGFRα was still detectable at 44 h of serum deprivation in the mutant Cbl transfectant, the level of phosphorylation showed a marked time-related decrease [Fig. 6, αTyr(P) blot, lanes 7 to 12]. Subsequent reimmunoblotting with anti-PDGFRα antibody revealed that slight variations in the levels of PDGFRα immunoprecipitated at different time points did not account for the steady decline in tyrosine phosphorylation signals. While these results did not fully rule out

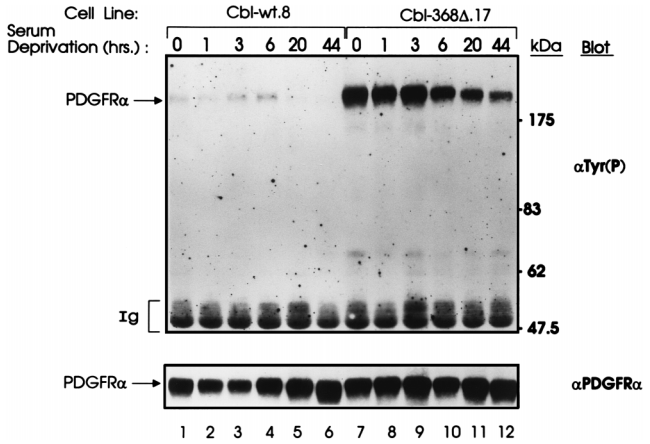

FIG. 6. Hyperphosphorylation of PDGFRα in NIH 3T3 cells transfected with oncogenic Cbl mutants is reduced by serum deprivation. A wild-type Cbl-expressing cell line (Cbl-wt.8) and a mutant Cbl-expressing cell line (Cbl-368Δ.17) were plated overnight in complete culture medium containing 10% FCS. Cells were then washed once with PBS and cultured in medium containing 0.5% FCS (deprivation) for the times indicated in hours (hrs.). Triton X-100 lysates were prepared at each time point, and a 250-μg lysate sample was subjected to immunoprecipitation with anti-PDGFRα antibody. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, immunoblotted with anti-Tyr(P) antibody, and detected with protein A-HRPO followed by ECL (upper panel). The membrane was stripped and reprobed with anti-PDGFRα antibody (lower panel). PDGFRα and the heavy chain of immunoglobulin (Ig) are indicated on left. Size markers are on the right.

constitutive activation of PDGFRα, the substantial decrease in PDGFRα phosphorylation upon serum starvation is consistent with enhanced responsiveness of PDGFRα in mutant Cbl-transfected cells toward exogenous ligands.

PDGF-AA induced phosphorylation of PDGFRα in mutant Cbl-transfected cells. To directly assess if PDGF-induced signaling events were different in cells expressing mutant versus wild-type Cbl proteins, we stimulated serum-starved cells with the selective PDGFRα ligand PDGF-AA (12). Cells were serum deprived for 48 h and then stimulated for various times with 20-ng/ml PDGF-AA. Lysates of unstimulated and PDGF-AA-stimulated cells were subjected to immunoprecipitation with anti-PDGFRα, followed by anti-Tyr(P) immunoblotting (Fig. 7).

Stimulation of serum-deprived cells with the PDGF-AA induced rapid tyrosine phosphorylation of PDGFRα in both wild-type (Cbl-wt.8) and mutant Cbl-transfected (Cbl-368Δ.17) NIH 3T3 cells (Fig. 7). At early time points (e.g., 1 to 10 min), the levels of PDGFRα tyrosine phosphorylation in the wild-type Cbl transfectant were comparable to or higher than in the mutant Cbl transfectant. However, at later points (e.g., 30 min and 3 h) the phosphotyrosine signal on PDGFRα was higher in the mutant Cbl transfectant than in the wild-type Cbl transfectant. These results suggest that PDGFRα in mutant Cbl transfectants is hyperresponsive to its ligand. This result, together with the decrease in phosphotyrosine signal on PDGFRα upon serum deprivation, suggests that a serum-derived and/or autocrine ligand contributes toward hyperphosphorylation of PDGFRα in cells cultured in regular serum-containing medium.

PDGF-AA can substitute for serum growth factors to maintain hyperproliferation of mutant Cbl-transfected NIH 3T3 cells. The above studies, demonstrating activation of an early PDGFRα signaling cascade in mutant Cbl transfectants, suggested that PDGF-dependent mitogenic activation may contribute to increased proliferation of mutant Cbl-transfected NIH 3T3 cells. To determine if this was the case, we cultured wild-type (Cbl-wt.8) cells and two mutant Cbl-transfected cell lines (Cbl-368Δ.6 and Cbl-N) either in complete medium (10% FCS) or in low-serum medium (0.5% FCS). Both mutant Cbl-transfected cell lines grew to a higher density in complete medium than did wild-type Cbl-expressing cells (Fig. 8A, solid bars). In each case, growth in low-serum medium resulted in a substantial decrease in cell numbers harvested at day 7, although a relatively higher degree of proliferation was still observed in mutant Cbl transfectants (Fig. 8A, stippled bars). Importantly, when low-serum medium was supplemented with PDGF-AA, a significant reconstitution of proliferative ability, approaching that seen in complete medium, was observed (Fig. 8A, striped bars). Thus, PDGF-AA can substitute for the serum-derived factors in maintaining hyperproliferation of mutant Cbl-transfected NIH 3T3 cells. The hyperresponsiveness of mutant Cbl-expressing cells was not the result of a wild-type Cbl-induced decrease in PDGF-AA responsiveness, as comparable PDGF-AA- and serum-induced proliferation was observed in wild-type Cbl-expressing and untransfected NIH 3T3 cells (Fig. 8B).

PDGF-AA induces stable PDGFRα-Cbl complexes in Cbl transfectants. Previous studies have demonstrated that PDGF-BB stimulation of NIH 3T3 cells induced tyrosine phosphorylation of Cbl, but PDGFR-Cbl complexes were not detectable (20). In view of abnormal phosphorylation of PDGFRα in mutant Cbl-transfected NIH 3T3 cells, we wished to assess if Cbl proteins and PDGFRα physically associate. In our initial studies of 3T3 transfectants grown in complete medium, long exposures of immunoblots revealed that anti-HA immunoprecipitates from Cbl-368Δ transfectants coimmunoprecipitated p185 whereas this association was much harder to detect in immunoprecipitates from wild-type Cbl transfectants (data not shown). To extend these initial observations, we carried out anti-Tyr(P) immunoblotting of anti-HA immunoprecipitates from mutant and wild-type Cbl transfectants stimulated with PDGF-AA (Fig. 9A).

These analyses indicated that both wild-type Cbl and mutant Cbl-368Δ became rapidly tyrosine phosphorylated upon PDGF stimulation (Fig. 9A, compare lane 1 with lane 2 and lane 8 with lane 9), although the basal level of Cbl-368Δ phosphory-

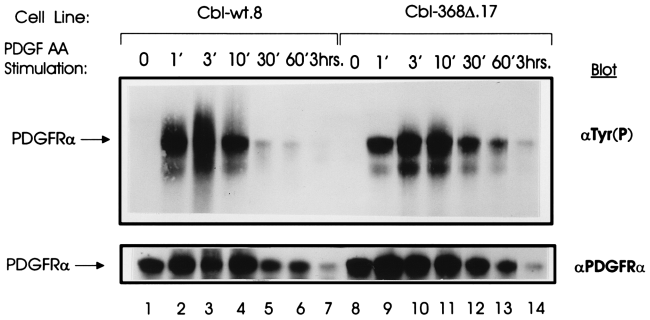

FIG. 7. Prolonged tyrosine phosphorylation of PDGFRα upon PDGF-AA stimulation of NIH 3T3 cells transfected with mutant Cbl. A wild-type Cbl-expressing cell line (Cbl-wt.8) and a mutant Cbl-expressing cell line (Cbl-368Δ.17) were plated overnight in complete culture medium containing 10% FCS, washed once with PBS, and serum deprived by culture for 2 days in medium containing 0.5% FCS. Cells were then stimulated with 20-ng/ml human recombinant PDGF-AA and lysed at the times indicated in minutes (′) or hours (hrs.). Immunoprecipitations were performed with anti-PDGFRα antibody from 250-μg Triton X-100 lysate samples. Isolated proteins were separated by SDS-PAGE, transferred to a PVDF membrane, probed with anti-Tyr(P) antibody, and visualized by using a protein A-HRPO conjugate and ECL detection (upper panel). The membrane was stripped and reprobed with anti-PDGFRα antibody (lower panel). PDGFRα is indicated on the left.

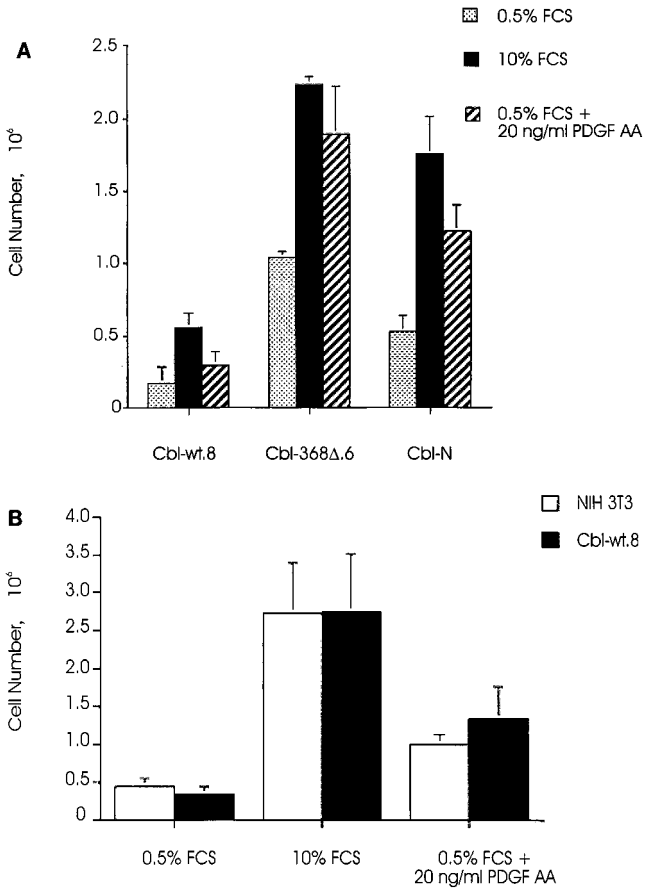

FIG. 8. PDGF-AA can substitute for serum growth factors to maintain hyperproliferation of NIH 3T3 cells expressing oncogenic mutant Cbl proteins. (A) A wild-type Cbl-expressing cell line (Cbl-wt.8) and two mutant Cbl-expressing cell lines (Cbl-368Δ.6 and Cbl-N) were plated in quadruplicate at a density of 5×10^3 cells per 25-cm^2 flask in medium containing 0.5% FCS. After 24 h, cells were switched to medium containing either 0.5% FCS, 10% FCS, or 0.5% FCS supplemented with 20-ng/ml PDGF-AA. The medium was changed on day 4, and the cells were released with trypsin-EDTA on day 7 and counted in a hemocytometer. Each bar represents mean ± the standard deviation of data pooled from three independent experiments. (B) Untransfected NIH 3T3 cells and a wild-type Cbl-expressing cell line (Cbl-wt.8) were plated in triplicate at a density of 2.5×10^4 cells per 25-cm^2 flask in medium containing 0.5% FCS. After 24 h, cells were switched to medium containing the indicated supplement(s) (0.5% FCS, 10% FCS, or 0.5% FCS plus 20-ng/ml PDGF-AA). The medium was changed on day 3, and the cells were released and counted on day 5. Each bar represents the mean ± the standard deviation of data pooled from two independent experiments. The difference in response to 20-ng/ml PDGF-AA between the NIH 3T3 and Cbl-wt.8 cell lines was statistically insignificant ($P > 0.05$).

lation was higher, as expected (Fig. 9A, compare lanes 1 and 8). More importantly, within 3 min of stimulation, anti-HA immunoprecipitates of the Cbl-368Δ cell line revealed prominent association of the mutant Cbl protein with other tyrosine-phosphorylated polypeptides, including p185 (Fig. 9A, lanes 9 to 14). p185 was identified as PDGFRα by its comigration with phosphorylated PDGFRα in anti-PDGFRα immunoprecipitates and immunodepletion with anti-PDGFRα antibody (Fig. 9C, compare lanes 7 and 8 and lanes 9 and 10). This association was also seen in the wild-type Cbl transfectant; however, it was more transient and substantially less p185 was detected (Fig. 9A, lanes 2 to 7). Control immunoprecipitations from Cbl-368Δ.6 cells with protein A-Sepharose beads or anti-CD8 antibodies revealed that coimmunoprecipitation of PDGFRα with anti-HA antibody was specific (Fig. 9C, lanes 1 to 4). Furthermore, anti-HA antibody failed to immunoprecipitate PDGFRα from untransfected NIH 3T3 cells stimulated with PDGF-AA (Fig. 9C, lanes 11 to 14). These results demonstrate that both the wild-type and mutant Cbl-368Δ proteins are tyrosine phosphorylated in response to PDGF-AA stimulation and become associated with PDGFRα.

The enhanced association of PDGFRα with Cbl-368Δ mutant polypeptides, which retains its proline-rich regions and is prominently phosphorylated, could be mediated by SH3/SH2 domain-containing adaptor proteins (such as GRB2 and Nck), via the recently identified N-terminal PTB domain of Cbl, or through both mechanisms. Given the oncogenicity of Cbl-N (which lacks C-terminal proline-rich regions), we focused on the role of this region in Cbl's association with PDGFRα.

First, we examined Cbl-N-transfected NIH 3T3 cells for Cbl-PDGFRα complex formation upon PDGF-AA stimulation. As seen in Fig. 9B, tyrosine-phosphorylated p185, corresponding to PDGFRα, was observed in anti-HA immunoprecipitates upon PDGF stimulation of the Cbl-N cell line (lanes 2 to 5). The level of association was substantially lower than that observed in the Cbl-368Δ cell lines (data not shown), suggesting that SH3/SH2 adapter-mediated association is likely to provide additional stability to Cbl-PDGFRα complexes. PDGF-AA stimulation also induced an increase in the tyrosine phosphorylation of the Cbl-N protein (Fig. 9B, lanes 2 to 4), although this has not been reproducibly observed. Thus, the N-terminal transforming region of Cbl undergoes PDGF-AA-inducible association with PDGFRα.

Direct in vitro binding of Cbl-N to PDGFRα and abrogation of this interaction by a G306E mutation. While the lack of proline-rich regions and predicted SH2 domain-binding sites in Cbl-N, together with the recent identification of a PTB domain within this region of Cbl, strongly suggested its direct association with PDGFRα, we wished to further characterize the mechanism of this association. First, we carried out in vitro lysate binding experiments by using GST-Cbl fusion proteins. For this purpose, lysates of an unstimulated or PDGF-AA-stimulated cell line (Cbl-368Δ.17) were incubated with either GST alone, GST–Cbl-N (aa 1 to 357), or GST–Cbl-N/G306E (which includes a Gly-to-Glu substitution corresponding to the loss-of-function mutation in the *C. elegans* Cbl homolog SLI-1). GST fusion protein-bound polypeptides and parallel anti-PDGFRα immunoprecipitates were subjected to immunoblotting with anti-Tyr(P) antibodies (Fig. 10A).

As shown in Fig. 10A, GST–Cbl-N preferentially bound to a 185-kDa phosphotyrosyl protein in a PDGF-AA-dependent manner (lanes 3 and 4). This polypeptide comigrated precisely with phosphorylated PDGFRα (Fig. 10A, compare lanes 4 and 8). Significantly, this binding was completely abrogated by a G306E mutation (Fig. 10A, lanes 5 and 6).

To assess if the binding between Cbl-N and PDGFRα was direct, we performed a filter-binding assay by using anti-Tyr(P) and anti-PDGFRα immunoprecipitates derived from unstimulated and PDGF-AA-stimulated cells. After initial probing of the filters with GST–Cbl-N or its G306E mutant form, filters were reprobed with anti-Tyr(P) antibody to visualize loaded phosphoproteins.

Figure 10B reveals that GST–Cbl-N binds directly to immunoprecipitated PDGFRα derived from PDGF-AA-stimulated cell lysates (lane 4). Binding to PDGFRα was relatively selective, as shown by lack of binding to other prominent phosphotyrosyl polypeptides present in anti-Tyr(P) immunoprecipitates (Fig. 9B, compare lane 2 with lane 6). Importantly, the G306E mutation completely abolished the direct binding of Cbl-N to PDGFRα (Fig. 9C, lanes 2 and 4). These results demonstrate the ability of Cbl-N to directly bind to PDGFRα via a mechanism that involves its evolutionarily conserved PTB domain.

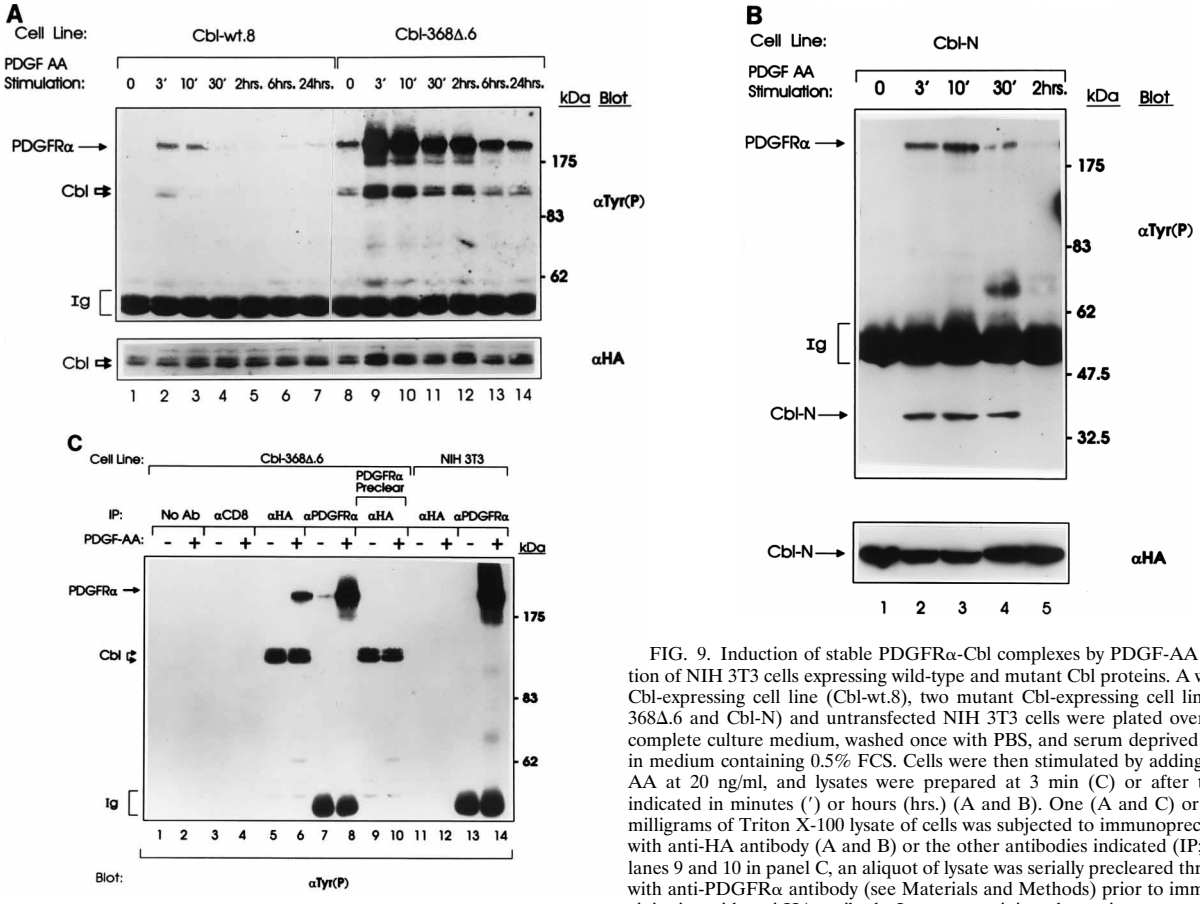

FIG. 9. Induction of stable PDGFRα-Cbl complexes by PDGF-AA stimulation of NIH 3T3 cells expressing wild-type and mutant Cbl proteins. A wild-type Cbl-expressing cell line (Cbl-wt.8), two mutant Cbl-expressing cell lines (Cbl-368Δ.6 and Cbl-N) and untransfected NIH 3T3 cells were plated overnight in complete culture medium, washed once with PBS, and serum deprived for 48 h in medium containing 0.5% FCS. Cells were then stimulated by adding PDGF-AA at 20 ng/ml, and lysates were prepared at 3 min (C) or after the time indicated in minutes (′) or hours (hrs.) (A and B). One (A and C) or two (B) milligrams of Triton X-100 lysate of cells was subjected to immunoprecipitation with anti-HA antibody (A and B) or the other antibodies indicated (IP; C). For lanes 9 and 10 in panel C, an aliquot of lysate was serially precleared three times with anti-PDGFRα antibody (see Materials and Methods) prior to immunoprecipitation with anti-HA antibody. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to PVDF membranes, immunoblotted with anti-Tyr(P) antibody, and visualized by using protein A-HRPO and ECL detection (A and B [upper panels] and C). The membrane was stripped and reprobed with anti-HA antibody (A and B, lower panels). A, B, and C represent separate experiments. PDGFRα, transfected Cbl proteins, and the heavy chain of immunoglobulin (Ig) are indicated on the left. Size markers are on the right.

A G306E mutation in Cbl-N abrogates mutant Cbl's effect on PDGFRα. In view of the failure of GST–Cbl-N/G306E to bind PDGFRα in vitro, we wished to determine its effect on PDGFRα in vivo. A pair of NIH 3T3 cells expressing comparable levels of HA–Cbl-N or HA–Cbl-N/G306E protein were derived by retroviral infection. In contrast to Cbl-N, the G306E mutant failed to induce morphologic transformation of NIH 3T3 cells (Fig. 11A) and cells expressing this mutant exhibited reduced proliferation compared to HA–Cbl-N transfectants, failed to form tumors in nude mice, and did not form colonies in soft agar (data not shown). Importantly, Cbl-N carrying the G306E mutation failed to induce hyperphosphorylation of PDGFRα, as revealed by anti-Tyr(P) immunoblotting of anti-PDGFRα immunoprecipitates (Fig. 11B, compare lanes 2 and 3). Thus, the ability of Cbl-N to interact with PDGFRα in vitro and to promote its hyperphosphorylation in vivo, and its transforming ability strictly require the integrity of the PTB domain.

DISCUSSION

The studies reported here provide evidence for a novel mechanism of oncogenesis by mutant forms of the Cbl proto-oncoprotein and, by implication, identify a potential physiological role for this polypeptide. The ability of the naturally occurring or engineered oncogenic mutants of Cbl to transform NIH 3T3 cells (2) provided an experimental system in which to discern if oncogenic mutants induce a common biochemical lesion linked to cellular proliferation. Here, we demonstrate that a feature common to three distinct Cbl oncoproteins is the ability to enhance PDGFRα tyrosine kinase signaling in NIH 3T3 fibroblasts. This enhancement was revealed by a constitutive increase in the phosphotyrosine content of a number of cellular polypeptides, including a 185-kDa polypeptide that we identified as PDGFRα. As a consequence of its hyperphosphorylation, PDGFRα showed enhanced association with a number of downstream signaling proteins, including the PI3-kinase p85 subunit, PLCγ1, GRB2, and SHC, which play important roles in mediating mitogenic response to PDGF (12, 44). Upregulation of PDGFRα signaling was observed in cells expressing a naturally occurring 17-aa deletion mutant form of Cbl (Cbl-70Z), an artificially derived Cbl protein with a deletion of a tyrosine close to the ring finger domain (Cbl-368Δ), and a truncated version of the normal polypeptide (Cbl-N) corresponding to Cbl sequences that are present in the viral oncogenic form of the protein (v-Cbl). Each of these mutant forms of Cbl have been previously shown to induce dramatic morphologic transformation of NIH 3T3 cells, and these transformed cells form tumors when implanted in nude mice and form colonies in soft agar (2). In contrast to the mutant forms of Cbl, expression of wild-type Cbl protein, which does not induce cellular transformation, did not augment the PDGFR signaling cascade.

Prior studies have revealed that Cbl oncoproteins with small

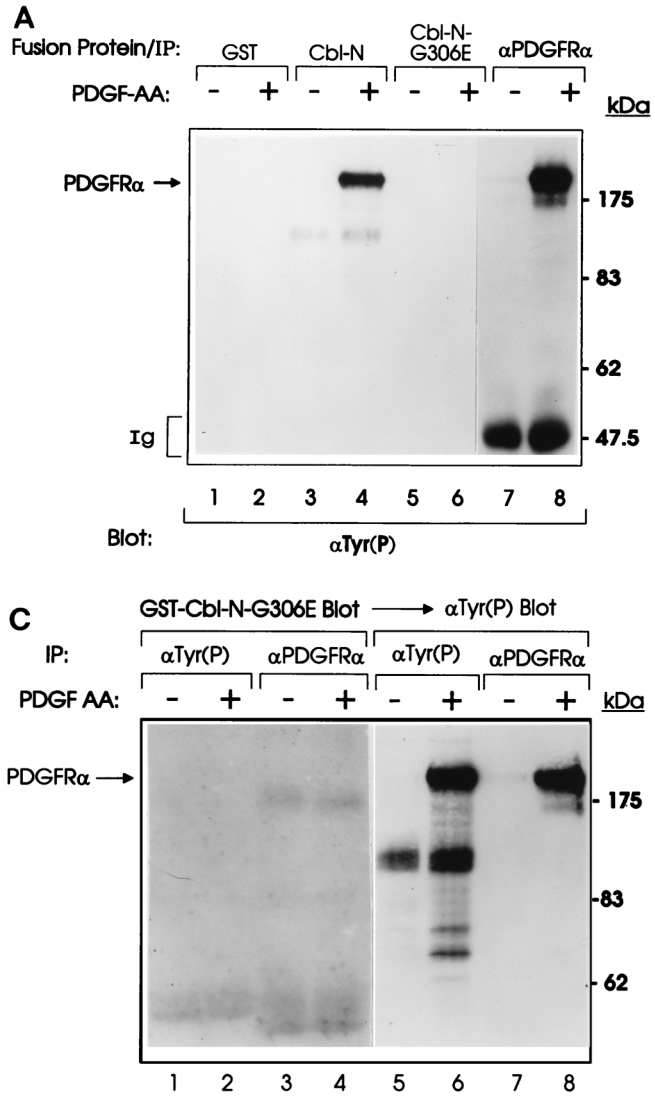

FIG. 10. Direct in vitro binding of Cbl-N to PDGFRα and abrogation of binding by a G306E mutation in Cbl-N. A mutant Cbl-expressing cell line (Cbl-368Δ.17) was plated overnight in complete medium containing 10% FCS, washed once in PBS, and serum deprived for 2 days in medium containing 0.5% FCS. Cells were then left unstimulated (minus lanes) or stimulated with 20-ng/ml PDGF-AA for 1 min (plus lanes), and Triton X-100 lysates were prepared for binding reactions. (A) In vitro lysate binding. Twenty-five micrograms of GST, GST–Cbl-N, or GST–Cbl-N/G306E noncovalently immobilized on glutathione-Sepharose was incubated overnight with 1 mg of Triton X-100 lysate. Anti-PDGFRα immunoprecipitation (IP) was carried out in parallel by using 250 μg of cell lysate. Bound polypeptides were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-Tyr(P) antibody followed by a protein A-HRPO conjugate and ECL detection. (B and C) Filter-binding assay. Anti-Tyr(P) and anti-PDGFRα immunoprecipitations from unstimulated (minus lanes) or PDGF-AA-stimulated (plus lanes) cell lysates were resolved by SDS-PAGE and transferred to a PVDF membrane. The membrane was then incubated with a 2.5-μg/ml concentration of eluted GST–Cbl-N (B) or GST–Cbl-N/G306E (C) and immunoblotted with an anti-GST monoclonal antibody. The immunoblot was visualized by using anti-mouse HRPO and ECL detection (left panel). Membranes were stripped and reprobed with anti-Tyr(P) antibody (right panel). PDGFRα and the heavy chain of immunoglobulin (Ig) are indicated on the left. Size markers are on the right.

deletions near the N-terminal boundary of the ring finger region (such as Cbl-70Z and Cbl-368Δ) undergo constitutive tyrosine phosphorylation in vivo (2). This finding has suggested one mechanism of Cbl oncogenesis, namely, that mutant forms of Cbl generate constitutive mitogenic signals by allowing increased recruitment of SH2 domain-containing signaling proteins. We and others have previously demonstrated that the p85 subunit of PI3-kinase and Crk adapter proteins interact selectively with tyrosine-phosphorylated Cbl via their SH2 domains and bring their associated proteins (such as the p85-associated PI3-kinase p110 subunit or the Crk SH3-bound C3G nucleotide exchange factor) into a ternary complex with Cbl (3, 9, 14, 19, 21, 24, 29, 32, 33, 37, 38). However, the known Crk SH2-binding site (Y774) and potential p85 SH2-binding sites (Y371 and Y731) are located C terminal to the truncation that generates the v-Cbl oncoprotein (equivalent to Cbl-N). The fact that Cbl-N is transforming in NIH 3T3 cells and, unlike Cbl-70Z, Cbl-368Δ, and Cbl-371Δ, does not show constitutive tyrosine phosphorylation (8, 28) suggested that oncogenesis mediated by mutant forms of Cbl may involve an alternative mechanism. Thus, our demonstration that a growth factor receptor tyrosine kinase is targeted by both Cbl-N and other Cbl oncoproteins provides a unifying biochemical basis of Cbl's oncogenic activity.

We propose that Cbl directly interacts with cellular tyrosine kinases via its N-terminal PTB domain and regulates their signaling function. This proposal is further supported by recent findings obtained with lymphocytes and myeloid cells demonstrating Cbl's association with ZAP-70/Syk tyrosine kinases—an association mediated by Cbl's PTB domain (28). Furthermore, Cbl's nematode homolog, SLI-1, has been identified as a negative regulator of the *C. elegans* EGFR homolog, LET-23 (23). Importantly, the loss-of-function mutation (G315E) that eliminates the negative regulatory effects of SLI-1 on LET-23 lies within the N-terminal region, which shows strong evolutionary conservation (23, 48). The mutation itself involves a conserved glycine located in a 21-aa stretch that is 80% identical between the two species (48). Dramatically, the corresponding mutation in human Cbl (G306E) results in loss of the ability of Cbl-N to bind to ZAP-70/Syk (28), PDGFRα (this study), and the mammalian EGFR (our unpublished data). Importantly, the G306E mutation of Cbl-N abrogates its transforming ability, enhancement of cellular proliferation, and, concomitantly, its ability to alter PDGFRα signaling. Together, these findings provide direct support for Cbl-N-mediated PTB domain-dependent regulation of cellular tyrosine kinases.

Presumably, the PTB domain-dependent Cbl interaction with

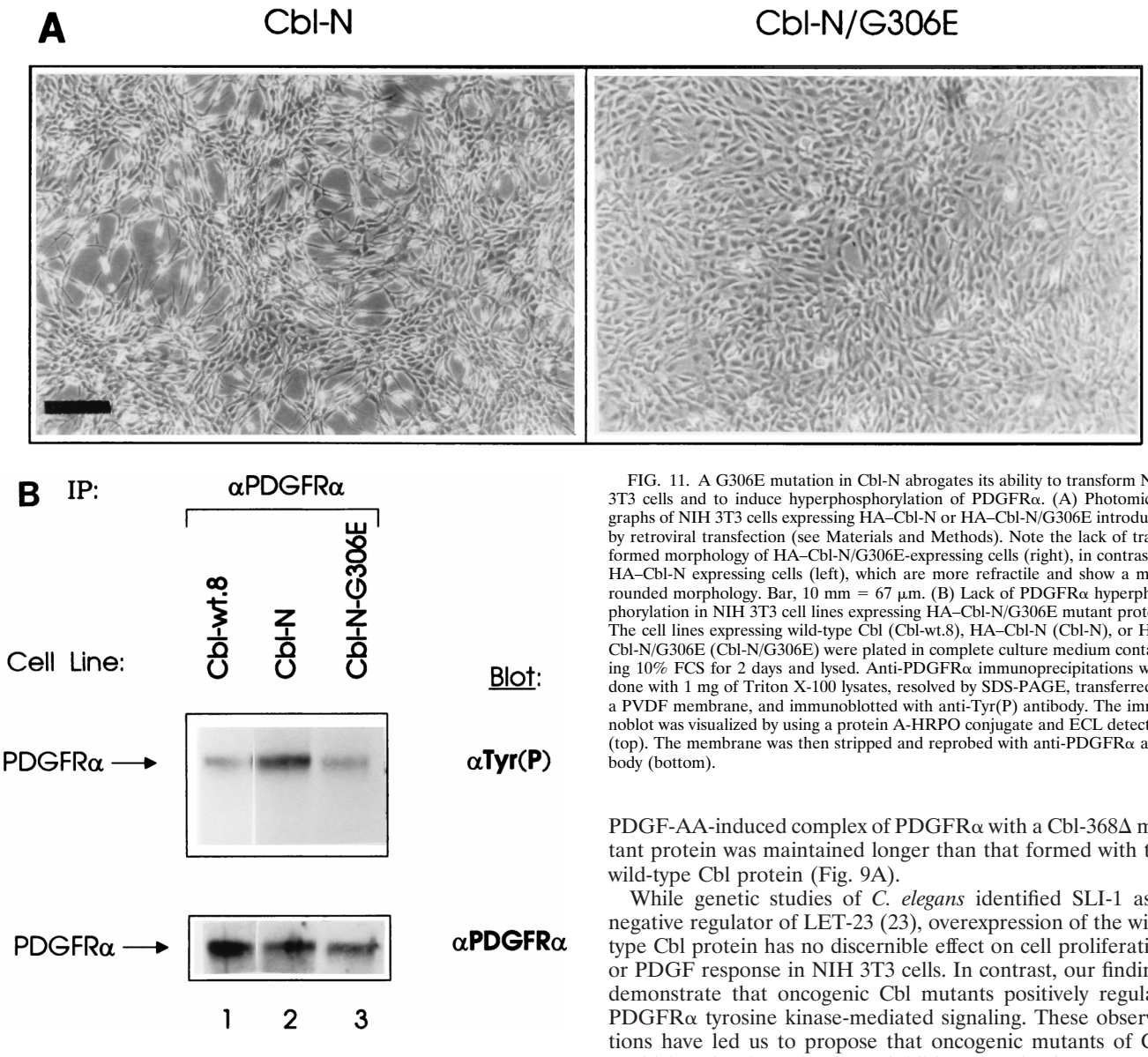

FIG. 11. A G306E mutation in Cbl-N abrogates its ability to transform NIH 3T3 cells and to induce hyperphosphorylation of PDGFRα. (A) Photomicrographs of NIH 3T3 cells expressing HA–Cbl-N or HA–Cbl-N/G306E introduced by retroviral transfection (see Materials and Methods). Note the lack of transformed morphology of HA–Cbl-N/G306E-expressing cells (right), in contrast to HA–Cbl-N expressing cells (left), which are more refractile and show a more rounded morphology. Bar, 10 mm = 67 μm. (B) Lack of PDGFRα hyperphosphorylation in NIH 3T3 cell lines expressing HA–Cbl-N/G306E mutant protein. The cell lines expressing wild-type Cbl (Cbl-wt.8), HA–Cbl-N (Cbl-N), or HA–Cbl-N/G306E (Cbl-N/G306E) were plated in complete culture medium containing 10% FCS for 2 days and lysed. Anti-PDGFRα immunoprecipitations were done with 1 mg of Triton X-100 lysates, resolved by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with anti-Tyr(P) antibody. The immunoblot was visualized by using a protein A-HRPO conjugate and ECL detection (top). The membrane was then stripped and reprobed with anti-PDGFRα antibody (bottom).

PDGFRα takes place in normal cells but may be either short lived or of low stoichiometry such that stable Cbl-PDGFRα complexes are maintained only briefly. Notably, ligand-induced complexes of wild-type Cbl with other tyrosine kinases, such as EGFR, ZAP-70, and Syk, are more readily detectable (16, 17, 20, 33), suggesting that the mechanism delineated here may be of general significance. The inability of wild-type Cbl to transform cells even when overexpressed and the lower abundance and relatively transient nature of wild-type Cbl-PDGFRα complexes in NIH 3T3 cells support the view that the Cbl-N region is masked in the wild-type protein. This may reflect a special conformation of the protein and/or the influence of intra- or intermolecular protein-protein interactions. We suspect that activation-associated events, such as tyrosine phosphorylation, allow the Cbl-N region to be transiently exposed for interaction with tyrosine kinases such as PDGFRα. Mutations, such as point deletions near the ring finger domain and truncations (as in Cbl-N) presumably unmask the N-terminal region and allow immediate access of its PTB domain to tyrosine-phosphorylated PDGFRα. Consistent with this view, a PDGF-AA-induced complex of PDGFRα with a Cbl-368Δ mutant protein was maintained longer than that formed with the wild-type Cbl protein (Fig. 9A).

While genetic studies of *C. elegans* identified SLI-1 as a negative regulator of LET-23 (23), overexpression of the wild-type Cbl protein has no discernible effect on cell proliferation or PDGF response in NIH 3T3 cells. In contrast, our findings demonstrate that oncogenic Cbl mutants positively regulate PDGFRα tyrosine kinase-mediated signaling. These observations have led us to propose that oncogenic mutants of Cbl could function by a dominant inhibitory mechanism. We suggest that binding of mutant Cbl proteins to PDGFRα prevents endogenous wild-type Cbl from binding and from exerting its inhibitory effect. This model is consistent with the inactivation of the effects of mutant Cbl protein by the G306E mutation. This proposal supports a negative regulatory function of both mammalian Cbl and *C. elegans* SLI-1. However, the effect of oncogenic mutant Cbl proteins observed here is compatible with a positive regulatory role of Cbl-N. It remains possible that SLI-1 and Cbl can mediate different regulatory functions either in the context of different tyrosine kinases or in different cell types.

It is likely that the mechanism delineated here represents the primary oncogenic mechanism for Cbl-N, which lacks other structural motifs (such as proline-rich sequences and tyrosine phosphorylation sites) yet leads to cellular transformation. However, constitutive complexes of SH2 domain-containing proteins (such as the PI3-kinase p85 subunit and Crk) with hyperphosphorylated Cbl and association with SH3 domain-containing proteins (e.g., GRB2 and Nck) mediated by Cbl's proline-rich regions may also contribute to cellular transfor-

mation by internal deletion mutant forms of Cbl that have an intact C-terminal region, such as Cbl-70Z and Cbl-368Δ. It is noteworthy that internal deletion mutant proteins (such as Cbl-368Δ and Cbl-70Z) are significantly more efficient at inducing anchorage-independent growth than is Cbl-N (2). While upregulation of signaling downstream of PDGFRα, a mitogenic growth factor receptor, is likely to contribute to oncogenesis, it is probable that mutant Cbl proteins target other tyrosine kinases. Use of PDGFRα mutant cell lines should allow assessment of the relative role of PDGFRα in mutant Cbl-induced NIH 3T3 transformation.

The biochemical mechanisms that underlie Cbl-N mediated alteration of PDGFRα signaling (and presumably of other cellular tyrosine kinases) remain to be defined. Reduction in PDGFRα phosphorylation upon serum deprivation and substitution of serum-dependent proliferation by PDGF-AA suggest that mutant Cbl proteins induce ligand hyperresponsiveness. However, it is possible that the association of mutant forms of Cbl also induces constitutive activation of PDGFRα, analogous to the BPV E5 protein-induced constitutive activation of PDGFRβ (31, 35). The residual tyrosine phosphorylation of PDGFRα in mutant Cbl-expressing cells after 44 to 48 h of serum deprivation is consistent with this possibility. More detailed analyses are required to fully address the role of constitutive activation versus enhanced ligand responsiveness in mutant Cbl-induced upregulation of PDGFRα signaling. Acute exposure of mutant Cbl-expressing cells to PDGF-AA did not show higher phosphorylation of PDGFRα at early time points, whereas phosphorylation was higher at later time points after stimulation, as well as in cells grown in regular medium. Therefore, it is possible that either PDGFRα kinase activity in mutant Cbl transfectants remains elevated or binding of Cbl's PTB domain protects crucial tyrosine residues within the cytoplasmic tail of PDGFRα against dephosphorylation. The global increase in PDGFRα phosphorylation, as shown by enhancement of its complexes with several SH2 domain-containing proteins, favors the former possibility. A regulatory influence of Cbl could also be exerted via an effect on activation-dependent PDGFRα trafficking or indirectly by regulation of cellular phosphatase machinery. Further analyses are required to delineate the precise mechanisms of Cbl-dependent regulation of PDGFRα signaling.

PTB domains were initially identified in SHC and IRS-1 and are presumed to facilitate the recruitment of intracellular signal-transducing proteins to tyrosine kinases (34, 45). While Cbl may interact with tyrosine kinases in a similar manner, our studies here and genetic studies with *C. elegans* suggest that Cbl's PTB domain may facilitate direct or indirect regulation of tyrosine kinase function. Interestingly, in each cell type examined so far, Cbl-N shows a preferential and direct interaction with a dominant tyrosine kinase linked to cellular differentiation or proliferation (ZAP-70/Syk, EGFR, and PDGFRα) (20, 28, this study, and unpublished data). Given that the precise boundaries of Cbl's PTB domain have not been delineated, it remains possible that Cbl-N contains additional functional domains relevant to tyrosine kinase regulation and that the PTB domain simply mediates tethering. In this regard, it is notable that both the SH2 and PTB domains characterized thus far are about 100 aa long (34, 45), compared to the 355-aa Cbl-N protein.

The present findings in the model system of fibroblast transformation, together with results from hematopoietic antigen receptor signaling and genetic studies with *C. elegans*, suggest that the targets of Cbl's oncogenicity in its natural host cells (such as pre-B cells; 27) are also likely to be tyrosine kinases that regulate cellular proliferation and differentiation. More importantly, these data suggest a physiological role for Cbl as a regulator of mammalian tyrosine kinases. We suggest that this regulatory role involves Cbl's PTB domain-mediated tethering to cellular tyrosine kinases. These studies set a stage for future analyses aimed at directly discerning the biochemical mechanism of Cbl's tyrosine kinase regulatory function.

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