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Award Number: DAMD17-99-1-9085

TITLE: Regulation of FAK Signaling in Mammary Epithelial Cells by Cbl Proto-oncogene Product

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REPORT DATE: January 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20020910 028

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2002	Annual Summary (31 Dec 00 - 30 Dec 01)

4. TITLE AND SUBTITLE
Regulation of FAK Signaling in Mammary Epithelial Cells by Cbl Proto-oncogene Product

5. FUNDING NUMBERS
DAMD17-99-1-9085

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Proliferation and differentiation of breast epithelial cells are regulated by the coordinated activation of the cellular tyrosine kinase machinery upon stimulation through growth factor receptors and extra-cellular matrix receptor-induced activation of focal adhesion kinase FAK. This proposal is designed to investigate a novel hypothesis that Cbl, a negative regulator of growth factor receptors, attenuates FAK-dependent growth signals in mammary epithelial cells. For this purpose, the Cbl interaction sites on FAK will be determined and the impact of mutations in these sites on the ability of FAK to mediate growth signals will be investigated. Given the recent findings that Cbl functions as an ubiquitin ligase towards tyrosine kinases, we are examining the possibility that Cbl regulates FAK signaling by targeting FAK or its signaling substrates for degradation. The work reported here describes initial characterization of FAK mutants that appear to be unable to interact with Cbl tyrosine kinase-binding domain. Together with mutant forms of Cbl that do not mediate ubiquitination, these studies will establish if FAK is a target of Cbl. The present studies, thus, aim to define novel strategies to down-regulate proliferation signals in breast cancer cells.

14. SUBJECT TERMS
breast cancer

15. NUMBER OF PAGES
40

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Regulation of FAK Signaling in Mammary Epithelial Cells by Cbl Proto-Oncogene Product

Introduction:

The experiments proposed in this application were designed to test a unique hypothesis that the proto-oncogene product Cbl down-regulates proliferation signals in mammary epithelial cells by concurrently targeting the focal adhesion kinase, FAK, in addition to its better defined ability to target growth factor receptors of the ErbB family. Genetic studies, initially in C. elegans and Drosophila systems and recently using mouse knock-outs, as well as extensive biochemical studies have established Cbl as a negative regulator of tyrosine kinases. At the time of this application, the tyrosine kinase-binding (TKB) domain of Cbl had been shown to be crucial for Cbl function, and initial pull-down experiments indicated that the TKB domain of Cbl interacts with FAK. These findings provided a rationale for the proposed analyses to identify the TKB domain binding sites on FAK and to introduce mutations in FAK to abrogate its ability to interact with the Cbl TKB domain in order to investigate the potential regulatory role of Cbl for FAK. Subsequent studies in our laboratory have also demonstrated that Cbl interacts with and regulates the function of Src-family kinases, which in turn are known to be important for the activation of FAK. Notably, the regulation of Src-family kinases by Cbl involves at least two types of interactions, one dependent on the Cbl TKB domain and a second one involving binding between the proline-rich region of Cbl and the SH3 domain of Src-family kinases. Thus, it became clear that Cbl might regulate FAK both directly as well as indirectly via its negative regulatory effects on Src-family kinases. Additional transfection approaches and analysis of cell lines from Cbl+/+ wildtype and Cbl-/- knockout mice further supported the original hypothesis that Cbl controls FAK signaling in mammary epithelial cells. Studies reported here have provided important clues about the potential role of the Cbl interaction sites on FAK in controlling its activity. These studies have also highlighted technical hurdles that need to be overcome to fully investigate the role of the novel Cbl-dependent FAK regulatory pathway in the control of mammary cell proliferation. Understanding the mechanisms of this novel biochemical pathway to control mammary epithelial cell proliferation and migration is likely to provide crucial insights into breast cancer pathogenesis and may help in the design of newer forms of treatment.

Body:

Studies carried out in the current reporting period helped further the training of the trainee, and pointed to a potentially significant role of the putative Cbl-binding site on FAK in controlling its function. However, some of the approaches that we attempted have met with technical difficulties. Nevertheless, the overall goals remain unchanged and the modifications of technical approaches currently underway are expected to help us achieve our goals by the end of the project period.

Previous studies using a GST fusion protein had indicated that the Cbl TKB domain could directly bind to FAK. Analysis of Cbl mutants co-transfected with FAK further strengthened this observation but also indicated that additional mechanisms for Cbl-FAK

association were likely, as the G306E mutation of Cbl did not reduce the association. As the Cbl TKB domain recognizes phospho-tyrosine-containing motifs on target proteins, we investigated the possible motifs on FAK that could mediate such an interaction. These studies showed that two tyrosine phosphorylation sites on FAK, pY397 and pY861, were the likely candidates for Cbl TKB domain binding. Previous studies have indicated that FAK Y397 is an auto-phosphorylation site and mediates the recruitment of Src-family kinases such as Src, Fyn and Yes, by providing a docking site for their SH2 domains (Sieg DJ et al. Nat. Cell Biol. 2:249-256, 2000; Nakamura K et al. Oncogene 20:2626-2635, 2001; Abu-Ghazaleh R et al. Biochem. J. 360:255-264, 2001). Once Src-family kinases are recruited, they enhance the phosphorylation of FAK on other sites: Y861 is a prominent in vitro Src-induced phosphorylation site as well as a major in vivo phosphorylation site on FAK (Abu-Ghazaleh R et al. Biochem. J. 360:255-264, 2001). Furthermore, phosphorylated Y861 functions to help in further recruitment of the Src-family kinases, although the mechanisms of this interaction are less clear (Abu-Ghazaleh R et al. Biochem. J. 360:255-264, 2001). Thus, these findings raised a strong possibility that the Cbl TKB domain binding to FAK may be SFK-dependent. We tested this possibility directly, using co-transfection of FAK and Fyn followed by pull-down assays. We have previously obtained the following vectors encoding the Myc-tagged FAK and its mutants from Drs. Wen-Cheng Xiong (University of Alabama, Birmingham, Alabama) and Dr. J. Thomas Parsons (University of Virginia, Charlottesville, VA): FAK wildtype; FAK-Y397F; FAK-Y861F; and FAK-Y397/861F. Here, we assessed the ability of these proteins to bind to the Cbl-TKB domain. In these experiments, the FAK proteins were expressed in 293T epithelial cells by themselves or together with activated Fyn (Fyn-Y528F). Notably, GST-Cbl-N was able to pull-down the wildtype FAK protein only when Fyn-Y528F was co-expressed (Fig. 1A). Importantly, FAK-Y397F (the mutant with a potential defect in SFK recruitment) and the FAK-Y861F mutant (the putative Cbl-TKB domain-binding site mutant) were defective in Cbl-TKB domain binding (Fig. 1A and B). Furthermore, mutational inactivation of the Cbl TKB domain abrogated its ability to pull down FAK (Fig. 1B).

Given these observations with FAK mutants, we pursued their further characterization in two complementary systems. In the first approach, we have begun their analyses in mammary epithelial cells, as proposed in the application. In a complementary approach, we have also carried out their analyses upon transfection in the cervical epithelial cancer cell line Hela, as a rapid screening tool since these cells are easy to transfect, express a functional endogenous EGFR, and their morphological features are highly conducive for two-color immuno-localization of transfected proteins with components of FAK signaling.

First, we tested the ability of EGF and TGFα, ligands for EGF receptor, to stimulate the human mammary epithelial cells. When HPV 16 E6/E7-immortlized 16A5 or hTERT-immortalized 76N-TERT cell lines were stimulated with EGF or TGFα, and their lysates analyzed by anti-phospho-tyrosine blotting, an expected time-dependent increase in tyrosine phosphorylation of a number of cellular proteins, including the EGFR, was seen (Fig. 2 and 3). Phosphorylation of these cellular proteins was related to ligand concentration (Fig. 4). Importantly, EGF as well as TGFα stimulation induced the phosphorylation of the Cbl TKB domain-docking site, pY1045, on EGFR (Fig. 5). However, EGF-induced phosphorylation was more sustained, whereas that induced by TGFα was more transient. Indeed, Cbl EGFR association was induced by both ligands, but was more sustained with EGF (Fig. 6).

Importantly, the sustained EGFR-Cbl association correlated with a more rapid degradation of EGFR upon EGF stimulation of human mammary epithelial cells (Fig. 7). Using the stimulation conditions established through these studies, we assessed the status of FAK phosphorylation in human mammary epithelial cell line 76N-TERT upon EGF stimulation. For this purpose, anti-FAK immunoprecipitations were carried out from lysates of un-stimulated and EGF-stimulated cells, and these were immunoblotted with antibodies that specifically recognize pY397 and pY861 (BioSource Inc.). As mammary epithelial cells grow as adherent monolayers attached to extra-cellular matrix, an expected substantial basal phosphorylation on FAK tyrosine 397 was observed prior to stimulation (Fig. 8, upper left panel). Notably, a progressive reduction in tyrosine phosphorylation on Y397 was observed with time, although it remained detectable throughout the time course. Thus, it is unlikely that pY397 would mediate inducible association of FAK with Cbl. Notably, Y861 showed a small but detectable level of phosphorylation, which was significantly enhanced by EGF stimulation; the enhancement was quick being noticeable within one minute and remained elevated as along as 90 minutes (Fig. 7, upper right panel. Immunoblotting with anti-FAK antibody demonstrated that differential phosphorylation in various lanes was not due to differences in FAK levels (Fig. 8, lower panels). The induction of FAK Y861 phosphorylation upon EGF stimulation strongly supports the likelihood of EGF-induced recruitment of Cbl to FAK in mammary epithelial cells.

Given the findings above, we initiated overexpression of wildtype FAK and its mutants in human mammary epithelial cells. As the pCMV-myc based plasmid expression vectors were already available, we transfected these into 16A5 mammary epithelial cells line, using the Fugene-6 transfection reagent (Roche Biochemicals). These included: pCMV-myc (vector control); FAK-WT; FAK-Y397F; FAK-Y861F; and FAK-Y397/861F. Transfectants were selected in G418 (neomycin-resistant) and stable lines were obtained for each transfectant. Anti-Myc immunoblotting of cell lysates, however, failed to show detectable expression of transfected proteins. Based on our subsequent discussions with our collaborator Dr. Vimla Band, an expert in mammary epithelial cell transfection systems, this negative result is likely to be due to lower efficiency of plasmid transfection methods in mammary epithelial cells. Based on her advice, we have now initiated the alternative approach of retroviral infection. Attempts to construct pMSCV-based retroviral constructs by sub-cloning FAK and its mutants, however, proved unsuccessful; a series of DNA analyses revealed this to be a result of the loss of 3' restriction enzyme sites initially used to clone FAK and its mutants in pCMV-myc vector. While the reasons for the loss of restriction sites remain unclear, our inability to simply cut and paste the FAK cDNA inserts in a retroviral vector necessitated alternative strategies. In one strategy, we have sub-cloned an untagged wildtype FAK (from a distinct plasmid expression vector available in the laboratory) into pMSCV-puro retroviral vector and tested if such a construct expresses the FAK protein. This construct was found to efficiently express FAK upon transient transfection in 293T cells (not shown). This construct will be used for retroviral transduction of mammary epithelial cells. If sufficiently high levels of FAK can be over-expressed, mutations of Y397 and Y861 will be introduced using site-directed mutagenesis. In an alternative strategy, PCR-based cloning is being attempted to generate pMSCV-puro retroviral expression constructs encoding myc-tagged FAK and its mutants; these constructs are obviously preferable as these can be selectively analyzed in transfected cells.

While our mammary epithelial cell transfection have been underway, we have also examined the Cbl TKB domain non-binding FAK mutants upon transfection in Hela cervical epithelial cancer cell line as these cells can be easily transfected and have been widely used to examine various aspects of EGFR function. In initial studies, we transiently transfected the WT FAK and its mutants into Hela cells, using the calcium phosphate co-transfection method. Fixed cells were immuno-stained with anti-myc antibody 9E10 to visualize the transfected FAK protein, and with Cy2-conjugated phalloidin to stain filamentous actin (Fig. 9). EGF stimulation of un-transfected cells as well as cells transfected with wildtype FAK or FAK-Y397F mutant showed comparable basal and EGF-stimulated patterns of actin cytoskeleton. In each case, un-stimulated cells showed a higher abundance of actin stress fibers, which was reduced by EGF treatment. In contrast, cells expressing the FAK-Y861F mutant exhibited markedly altered morphology with rounded, start-shaped or smaller cells with short processes; neighboring un-transfected cells did not exhibit such changes. Notably, these effects were noted both basally and in EGF-stimulated cells. Importantly, the cells with altered morphology often showed a high concentration of actin near the cell boundary and loss of stress fibers. This pattern is reminiscent of that observed in FAK-/- cells (Sieg D. et al. J. Cell Sci. 112:2677-2691, 1999) (Fig. 9D and E) indicating an important role of phosphorylation on Y861 in regulating FAK function. These initial findings will be followed up, and provide further impetus to analyze the FAK-Cbl interaction in mammary epithelial cells.

Given the essential role of Src-family kinases in FAK-dependent cell migration (Klinghoffer RA, et al. EMBO J. 18:2459-2471, 1999), and the ability of Cbl to downregulate Src-family kinases (Andoniou et al. Molecular and Cellular Biology, 20:851-867, 2000), we have hypothesized that Cbl might influence FAK signaling indirectly, via ubiquitin-dependent degradation of Src-family kinases. Therefore we have initiated studies to investigate how Cbl regulates Src-family kinases. In collaboration with other members of the laboratory, this work has revealed that Cbl induces the degradation of Src-family kinases Fyn and Lck by their ubiquitination-dependent targeting to the proteasome (see attached publications #). The mutant Src-family kinases deficient in Cbl-dependent regulation are currently being generated, and will provide additional reagents to test the possibility that in vivo interaction between Cbl and FAK may be mediated indirectly via Src-family kinases.

Overall, the recent studies provide strong support for the role of Cbl binding sites on FAK in its function, but also have highlighted the need for modifications of our technical approaches. These insights will be taken into account in order to achieve the goals proposed in the initial application.

Key Research Accomplishments:

- Established the mammary epithelial cell stimulation conditions to induce FAK phosphorylation on the Cbl TKB domain-binding site pY861.

- Established retroviral FAK expression construct.

- Demonstrated a defect in actin cytoskeleton upon transfection of Cbl non-binding FAK mutants.

- Established that Cbl regulation of Src-family kinases is mediated by ubiquitin-dependent proteasome targeting.

Reportable Outcomes:

Publications:

- Rao N, Ghosh AK, Zhou P, Ota S, Andoniou CE, Douillard P, Band H. An essential role of ubiquitination in Cbl-mediated negative regulation of the Src-family kinase Fyn. **Signal Transduction** 2002; 1-3:1-11.

- Rao N, Miyake S, Douillard P, Dodge I, Fernandes N, Druker B, Band H. Negative regulation of Lck tyrosine kinase by Cbl ubiquitin ligase. **Proc. Natl. Acad. Sci. USA.** 2002; 99:3794-3799.

Reagents:

- Retroviral FAK expression construct pMSCV-puro-FAK.
- Mutant Fyn and Lck constructs with defined domain mutations.

Funding applied for based on this work:

The work carried out under this award is part of the background and preliminary studies for an NIH RO1 application (Molecular Control of EGF Receptor Down-Regulation) submitted by Dr. Band to the NIH in February 2002; this application is under review in the June/July 2002 review cycle.

Manuscripts included:

- Rao N, Ghosh AK, Zhou P, Ota S, Andoniou CE, Douillard P, Band H. An essential role of ubiquitination in Cbl-mediated negative regulation of the Src-family kinase Fyn. **Signal Transduction** 2002; 1-3:1-11.

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Conclusions:

In conclusion, our newer results in both model cell systems and mammary epithelial cells indicate that EGFR-mediated cellular activation promote an interaction between Cbl and FAK. In addition, initial studies demonstrate that mutation of Cbl-binding site on FAK alters its function in remodeling actin cytoskeleton. However, technical difficulties were encountered with the expression of FAK and its mutants in mammary epithelial cells; alternative strategies to address these problems have been initiated. The reagents and insights developed here should allow us to

accomplish our stated goals to elucidate the role of Cbl in regulating FAK signaling in mammary epithelial cells. Given the critical role of FAK signaling in growth factor and extra-cellular matrix-dependent cellular proliferation and differentiation, the findings presented here are likely to be of general significance for other cell types.

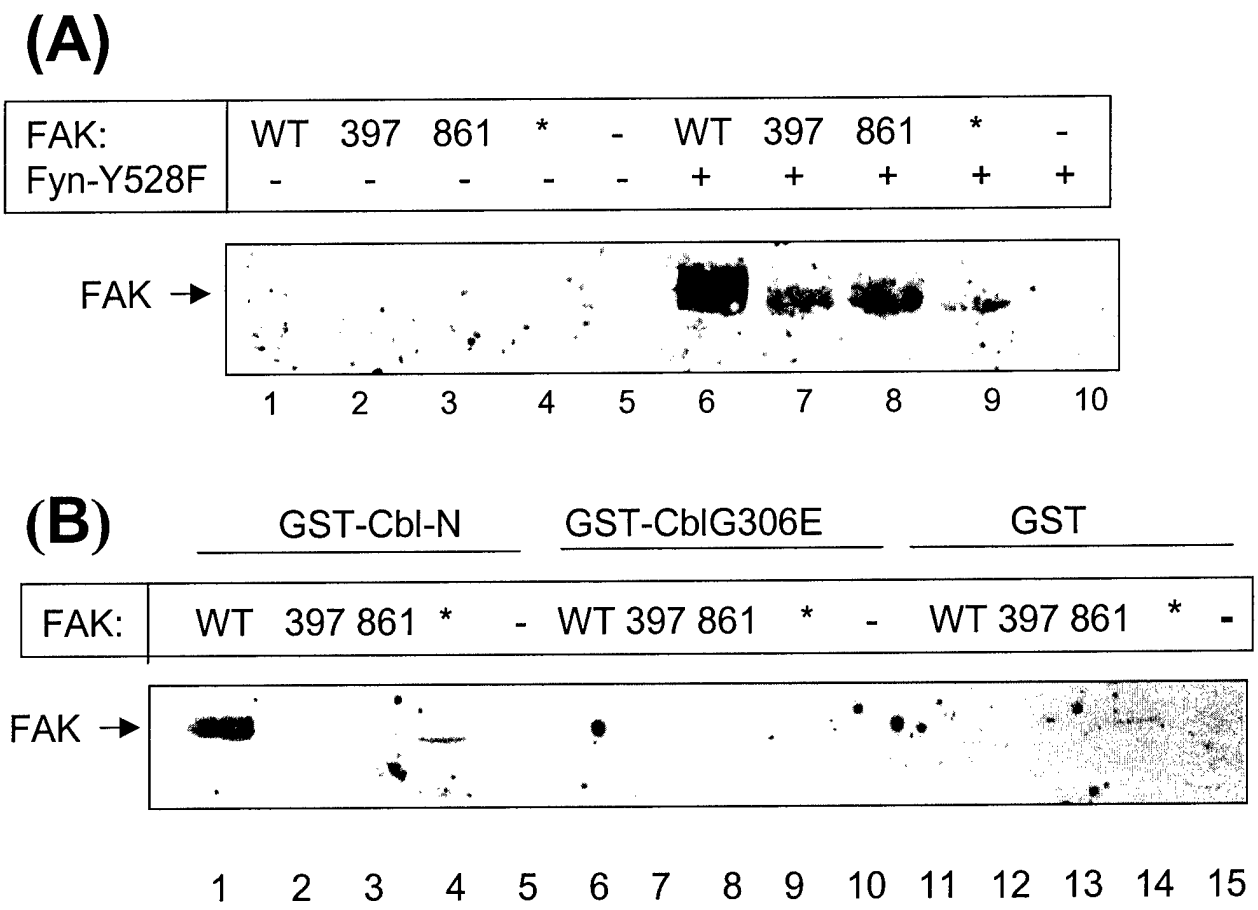


Fig. 1. Src-family kinase-dependent binding of Cbl TKB domain to wildtype FAK and abrogation of binding by mutation of FAK phosphorylation istes Y397 and Y861. **(A)** 293T cells were transfected with 0.3μg of a pAlterMAX construct encoding the activated Src-family kinase Fyn (Fyn-Y528F) or a vector control, together with 0.3μg of WT FAK, FAK-Y397F, FAK Y861F, or FAK Y397F/861F (denoted by an asterisk '*'). 40 hours post-transfection, lysates were prepared in RIPA buffer, and 1.5 mg aliquots of lysate protein were incubated with 20μg of GST-Cbl-N fusion protein for 2 hours. After extensive washing, binding reactions were resolved by SDS PAGE followed by anti-FAK immunoblotting. **(B)** Transfection, cell lysis and binding reactions were carried out as in A, with the exception that GST and GST-Cbl-N-G306E (TKB domain mutant) binding reactions were analyzed concurrently. FAK band is indicated by an arrow. Note that no binding to FAK is observed if Fyn is not co-expressed (A) and that Y397F and Y861F mutations markedly reduce binding to Cbl TKB domain, whereas the binding is essentially abrogated by mutations of both tyrosine residues.

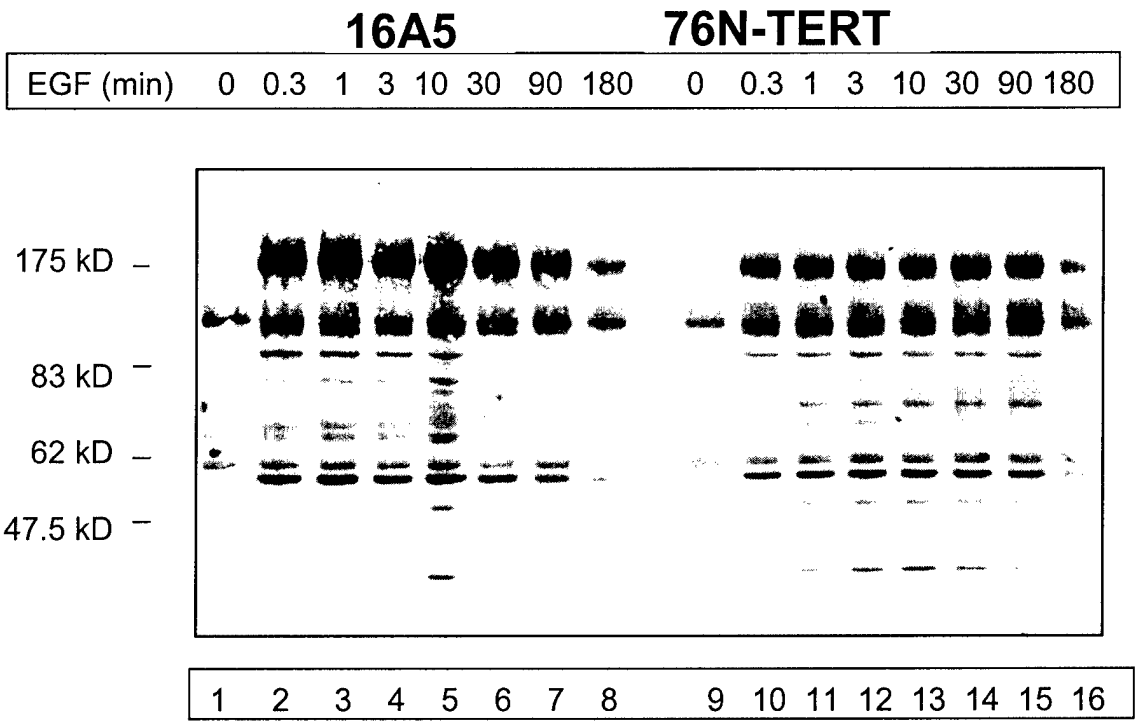


Fig. 2. Induction of protein tyrosine phosphorylation upon EGF treatment of 16A5 and 76N-TERT mammary epithelial cell lines. The cells were plated in DFCI-medium and then starved for 48 hours by growth in D3 medium. The cells were then left untreated (time 0) or treated with EGF (100 mg/ml) for 0.3, 1, 3, 10, 30, 90 or 180 min, and cell lysates were prepared. Equal aliquots (50μg) of lysate protein from each sample were resolved by SDS PAGE and transferred to a PVDF membrane. The membrane was immunoblotted with anti-Tyr (P) antibody 4G10.

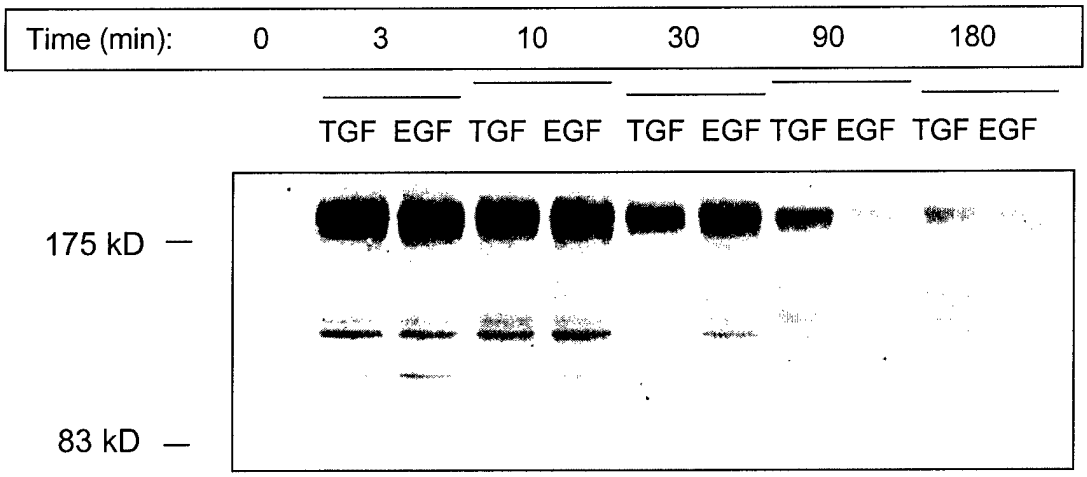


Fig. 3. Time course of protein tyrosine phosphorylation induced by TGF-alpha or EGF treatment of 76N-TERT mammary epithelial cells. The cells were plated in DFCI-1 medium and then starved of EGF by growth in D3 medium for 48 hours. The cells were then left untreated (time 0) or treated with TGF alpha or EGF (40 ng/ml) for 3, 10, 30, 90 and 180 min. Cell lysates were prepared and equal aliquotss (50 μg) of lysate protein from each sample were resolved by SDS PAGE and transferred to a PVDF membrane. The membrane was immunoblotted with anti-Tyr (P) antibody 4G10.

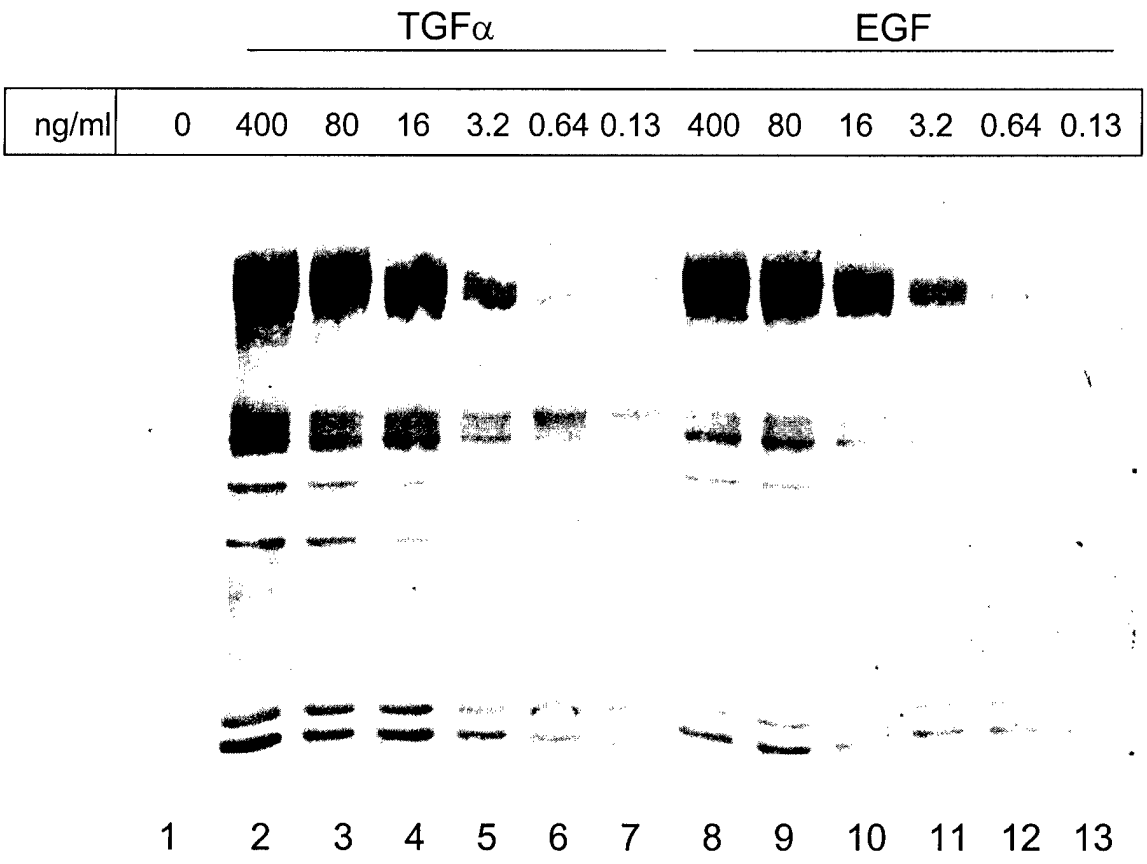


Fig. 4. Dose response of TGFα or EGF-induced protein tyrosine phosphorylation in 76N-TERT mammary epithelial cells. Stimulation with the indicated final concentrations of ligands (ng/ml) was performed as in Fig. 3 for 10 minutes. Cell lysis and anti-phosphotyrosine (4G10 antibody) immunoblotting was as in Fig. 3.

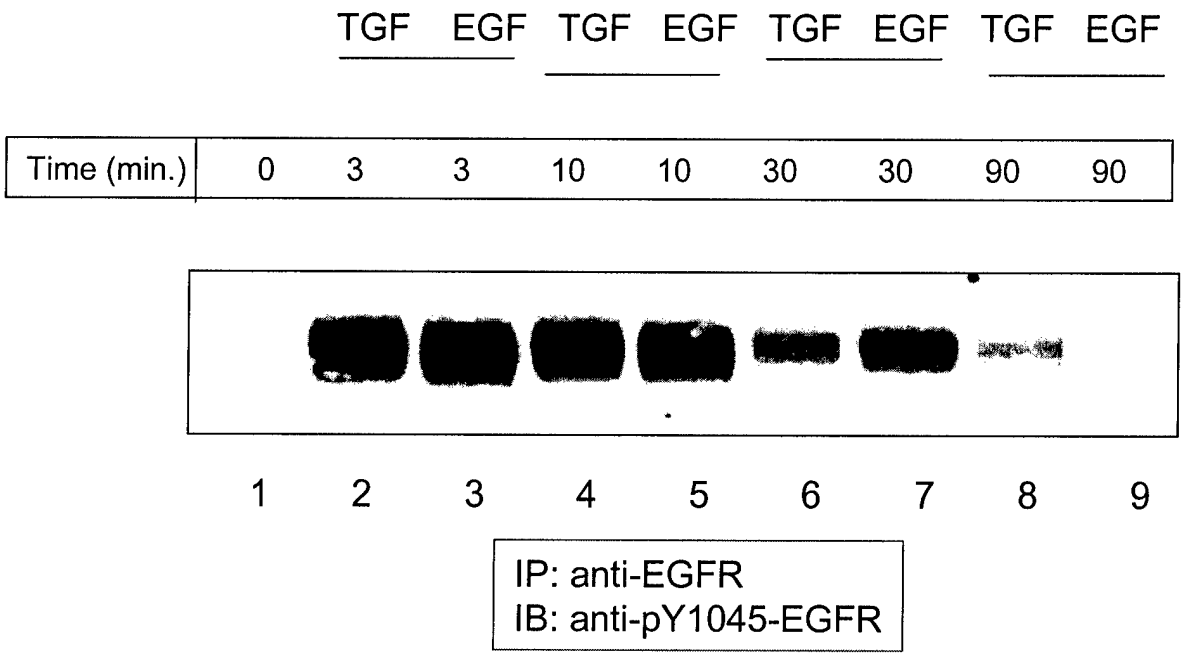


Fig. 5. Induction of Cbl TKB domain binding site (pY1045) on EGFR upon TGFα or EGF treatment of 76N-TERT mammary epithelial cells. The cells were starved for 2 days and then stimulated for the indicated time points with 150 ng/ml TGFα or EGF. Cell lysates were prepared in 1%Triton X-100 lysis buffer, and 200μg aliquots of lysate protein were subjected to immunoprecipitation with anti-EGFR antibody 528. The immunoprecipitated EGFR was immunoblotted with a rabbit phospho-specific antibody adirected against pY1045 (Cell Signaling Technology, Inc.).

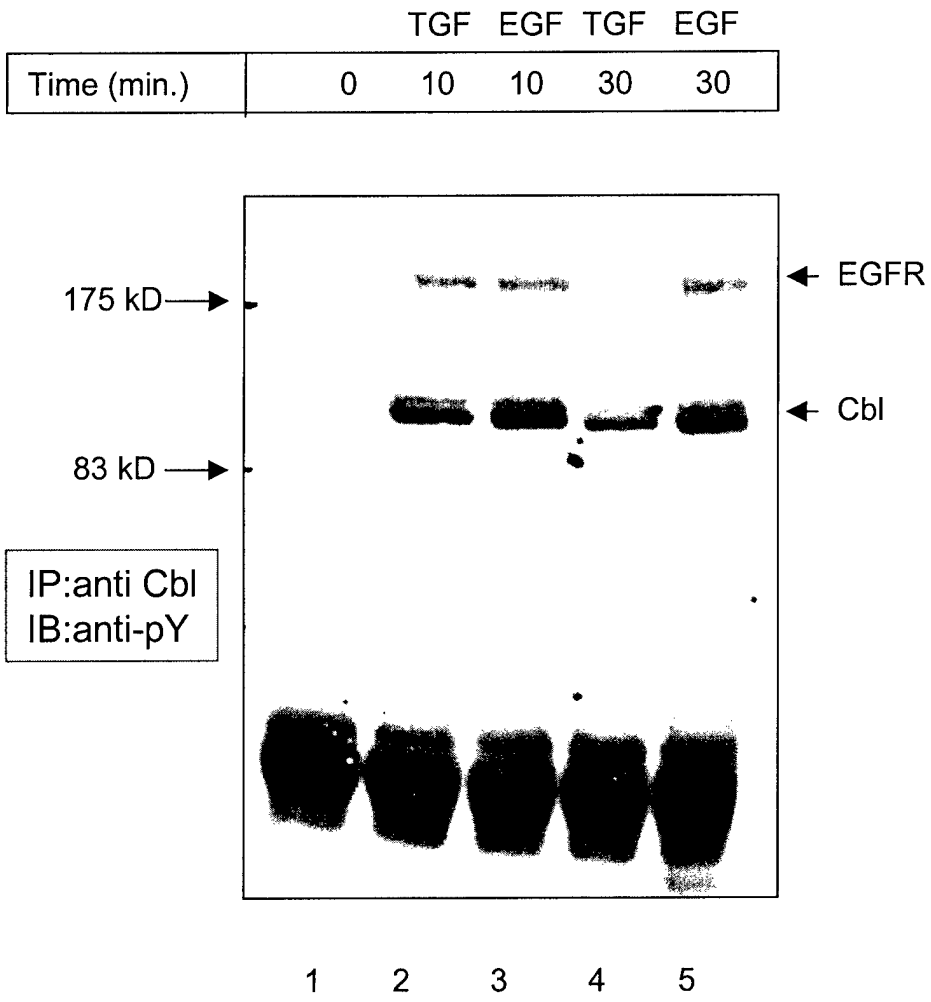


Fig. 6. Differential association between Cbl and EGFR following EGF versus TGFα treatment of 76N-TERT mammary epithelial cells. The cells were starved for 2 days, and either left unstimulated (0 min.) or stimulated for 30 min. with 150 ng/ml EGF or TGFα. Cell lysates were prepared in 1% Triton X-100 lysis buffer, and 1mg of aliquots of lysate protein were subjected to immunoprecipitation with an anti-Cbl antibody (PRAG-1). The immunoprecipitates were resolved by SDS PAGE followed by anti-phospho-tyrosine (4G10 antibody) imunoblotting.

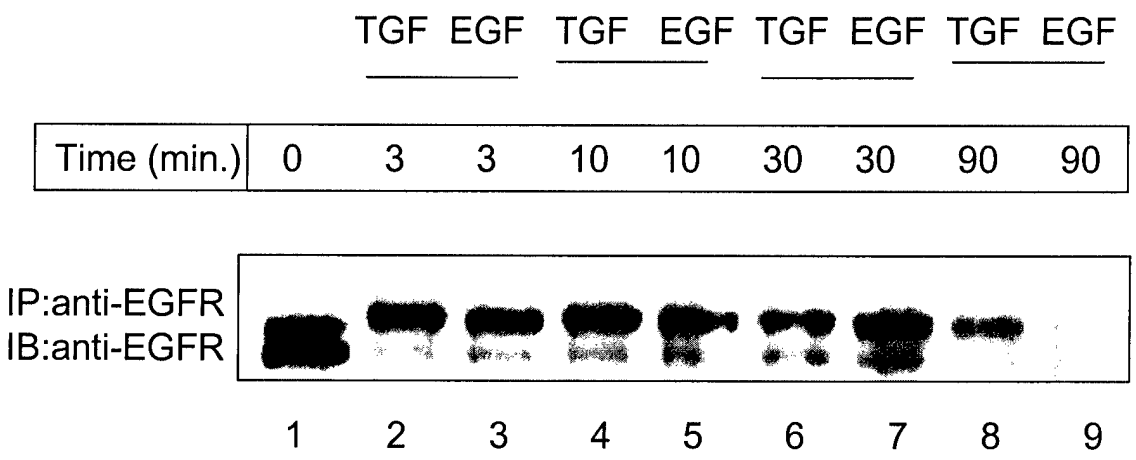


Fig. 7. Differential EGFR degradation induced by TGF α versus EGF treatment of 76N-TERT human mammary epithelial cell. The cells were starved for 2 days and then stimulated for the indicated time points with 150 ng/ml TGF α or EGF. Cell lysates were prepared in 1% Triton X-100 lysis buffer, and 300μg aliquots of lysate protein were subjected to immunoprecipitation with anti-EGFR antibody 528 followed by immunoblotting with anti-EGFR antibody sc003 (Santa Cruz Biotech.).

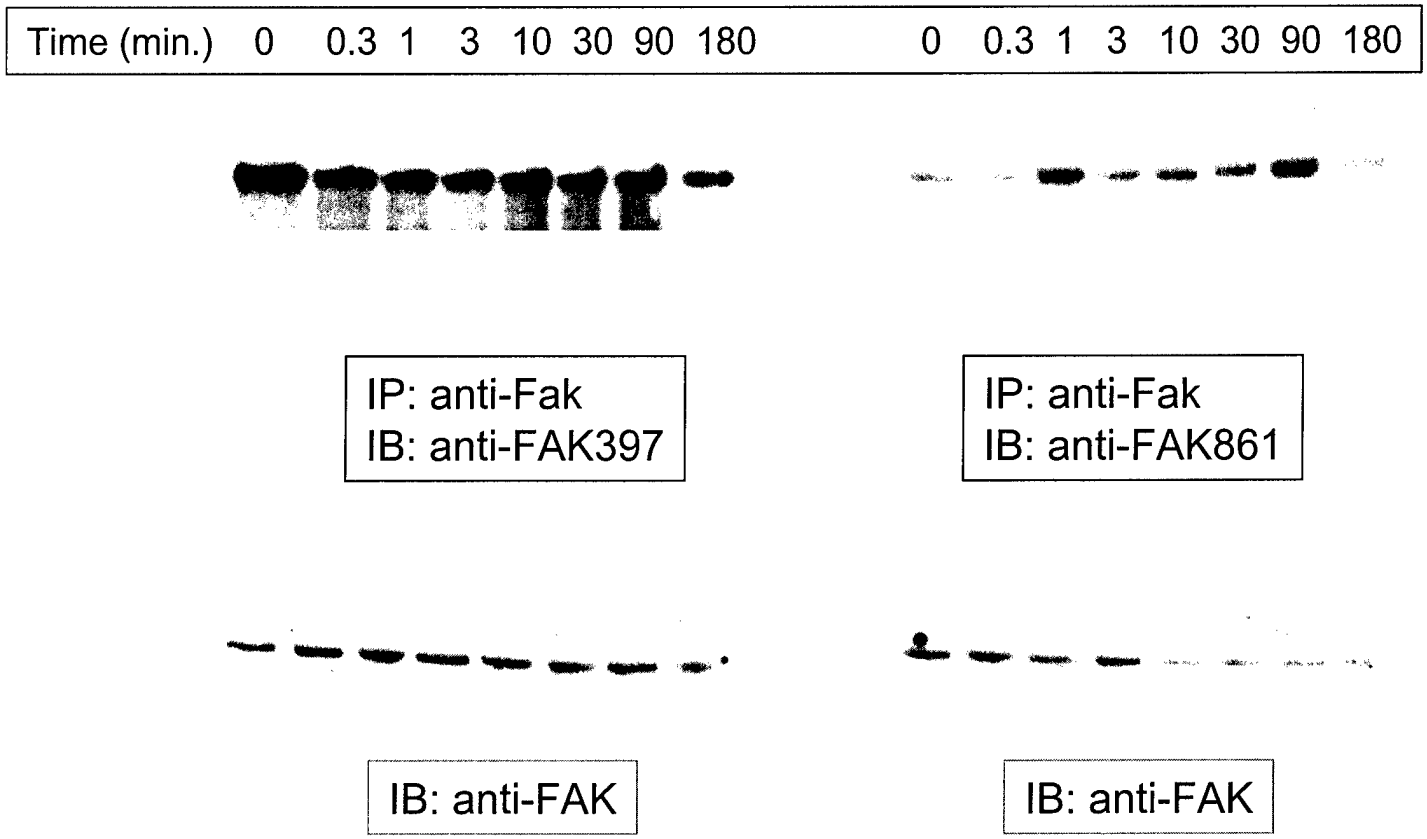


Fig 8. Analysis of FAK phosphorylation on tyrosine 397 and 861 upon EGF stimulation of 76N-TERT human mammary epithelial cell. The cells were starved for 2 days and then stimulated with 100 ng/ml EGF for the indicated time points. The cell lysates were prepared in RIPA lysis buffer, and 200μg aliquots of lysate protein were subjected to immunoprecipitation with an anti-FAK antibody followed by immunoblotting with phospho-specific rabbit antibodies directed at pY397 or pY861 (Biosource Inc.).

Fig. 9. Effect of wildtype versus mutant FAK expression on actin cytoskeleton in Hela cells. Hela cells were plated on glass coverslips overnight, and transfected with 2μg of pCMV-myc constructs encoding the Myc-tagged wildtype FAK (WT) or Y397F, Y861F, Y397/861F mutants. After 36 hours, the cells were starved for 6 hours and then stimulated with 100 ng/ml EGF for the indicated time points. The cells were then fixed in 3.7% formaldehyde/PBS for 20 min. and treated with saponin to permeabilize the cells. .Immuno-fluorescence staining was carried out with anti-Myc 9E10 antibody and anti-mouse Cy3 (red) to detect transfected FAK proteins. Cells were concurrently stained with phalloidin-Cy2 conjugate (green) to visualize the F-actin. Anti-myc staining of untransfected cells was completely negative and is not shown. Note the altered morphology and actin-staining of cells transfected with FAK-Y861F (D) or FAK-Y397/861F mutant (E).

Fig. 9A. Untransfected cells

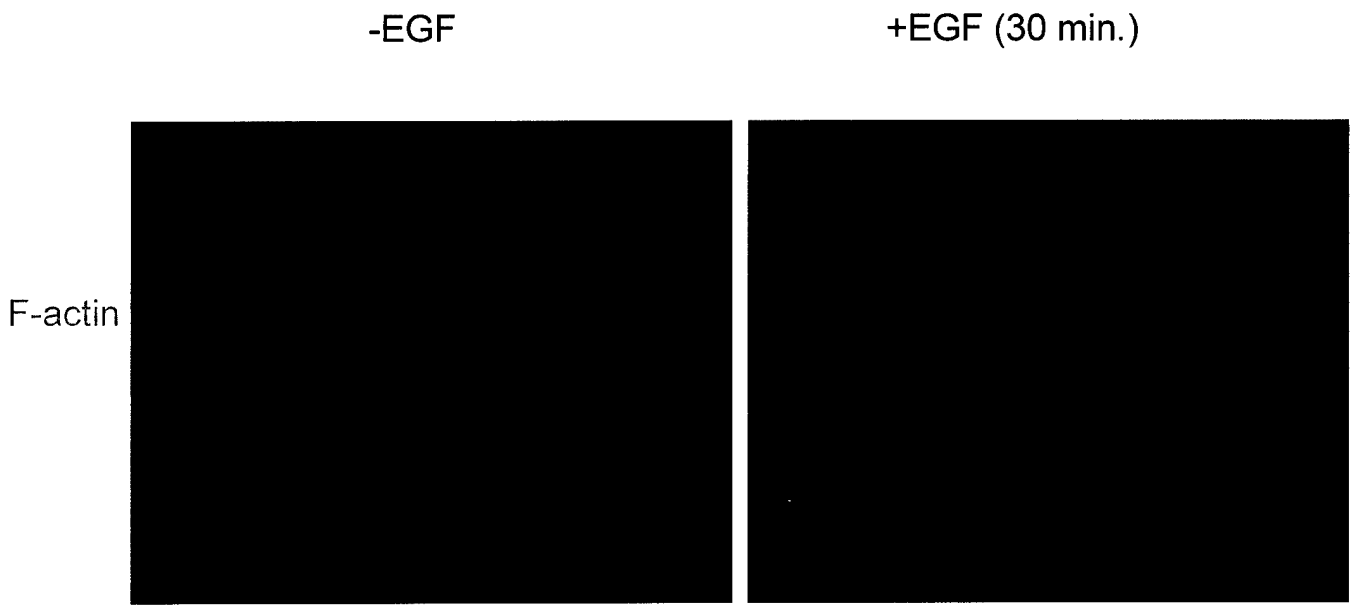


Fig. 9B. FAK WT-transfected

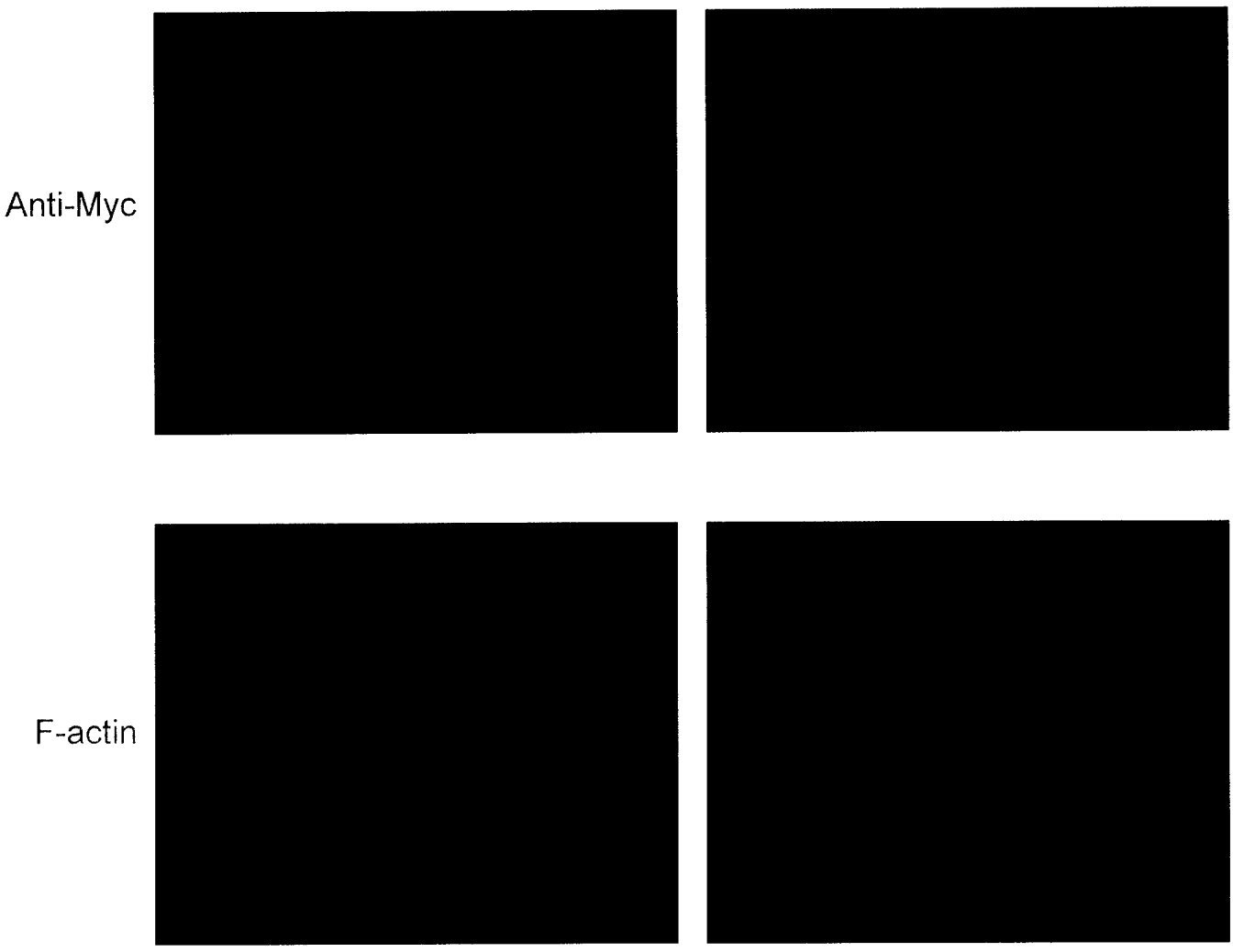


Fig. 9C. FAK-Y397F-transfected

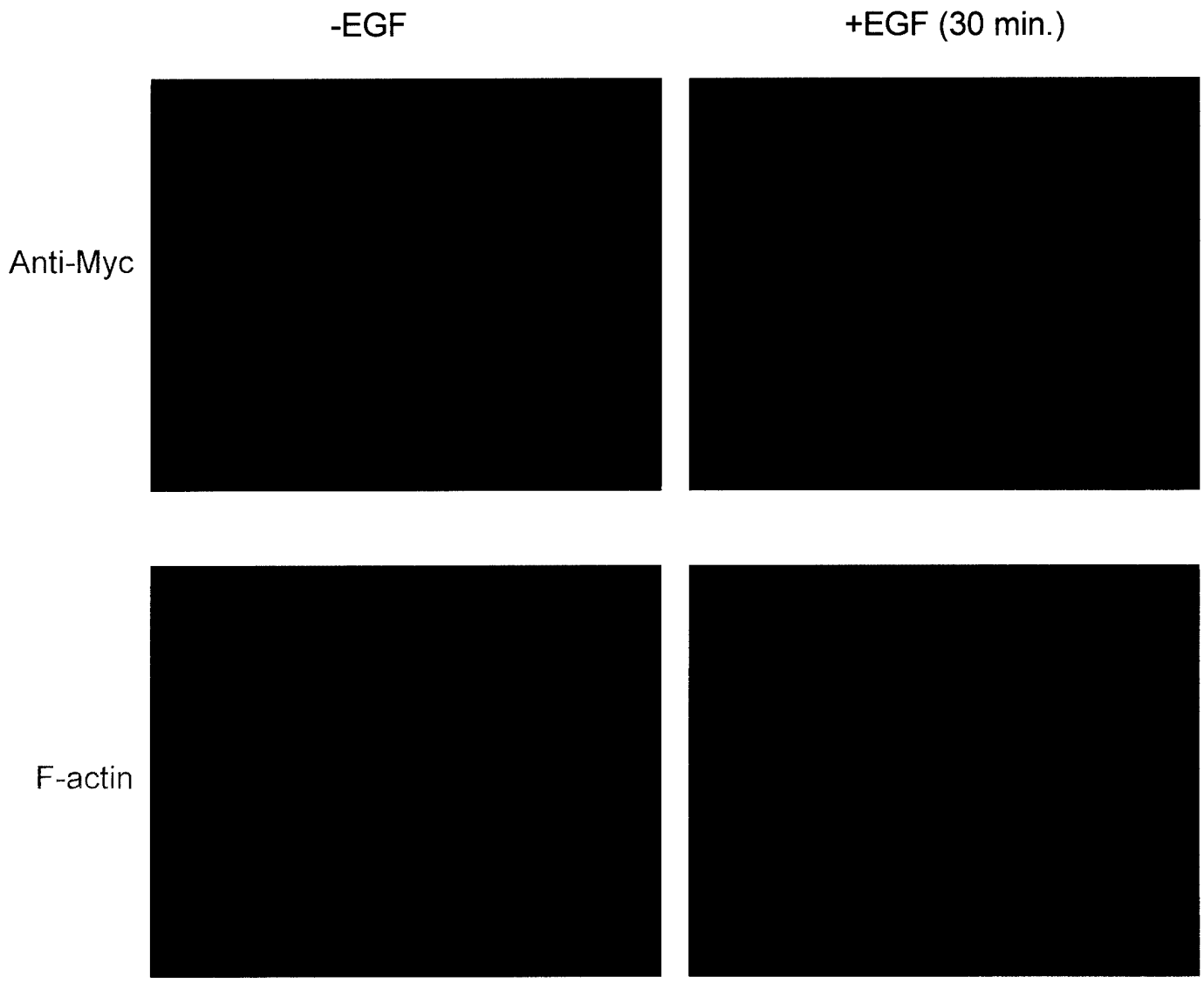


Fig. 9D. FAK-Y861F-transfected

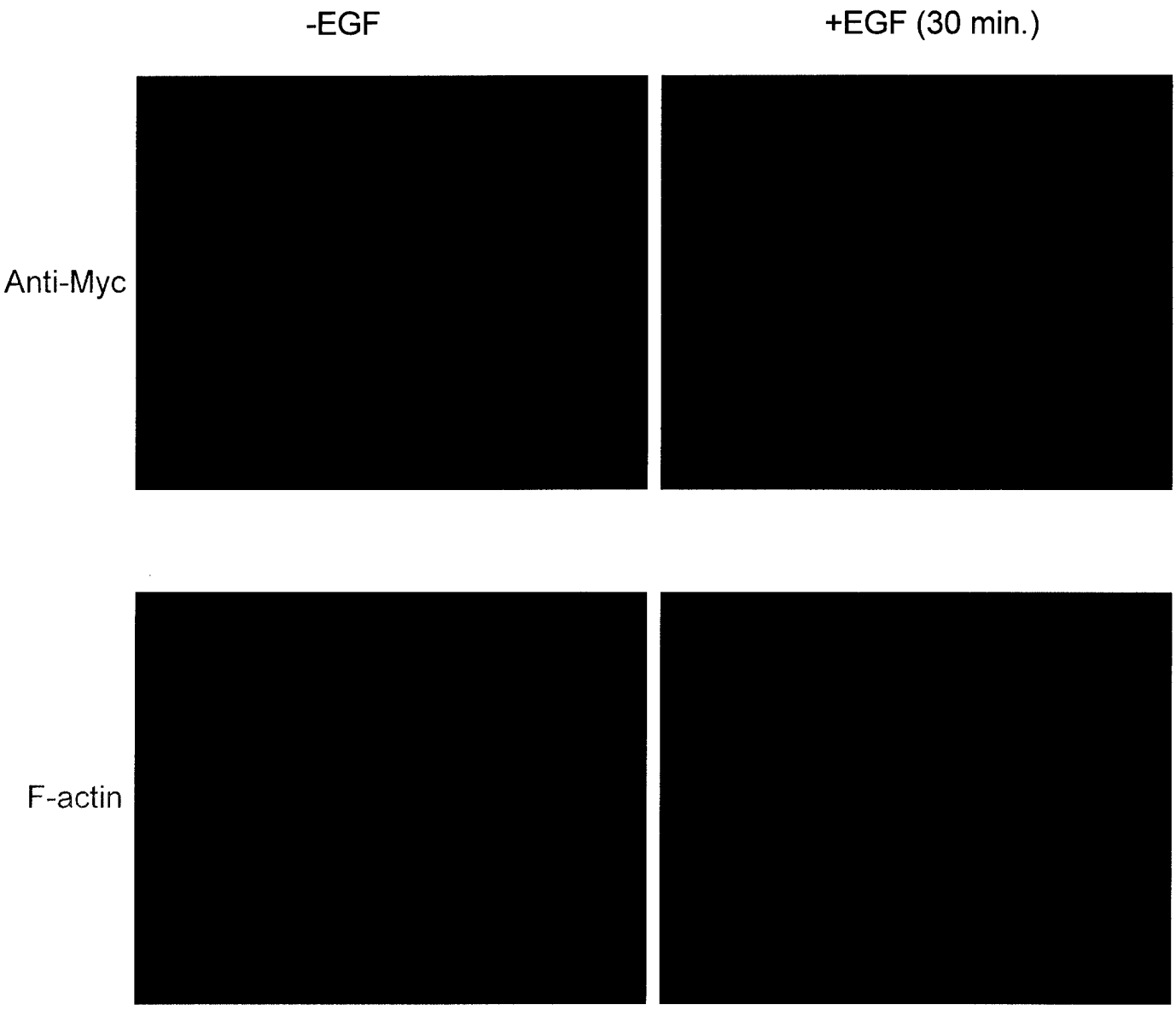


Fig. 9D (Contd). FAK-Y861F-transfected

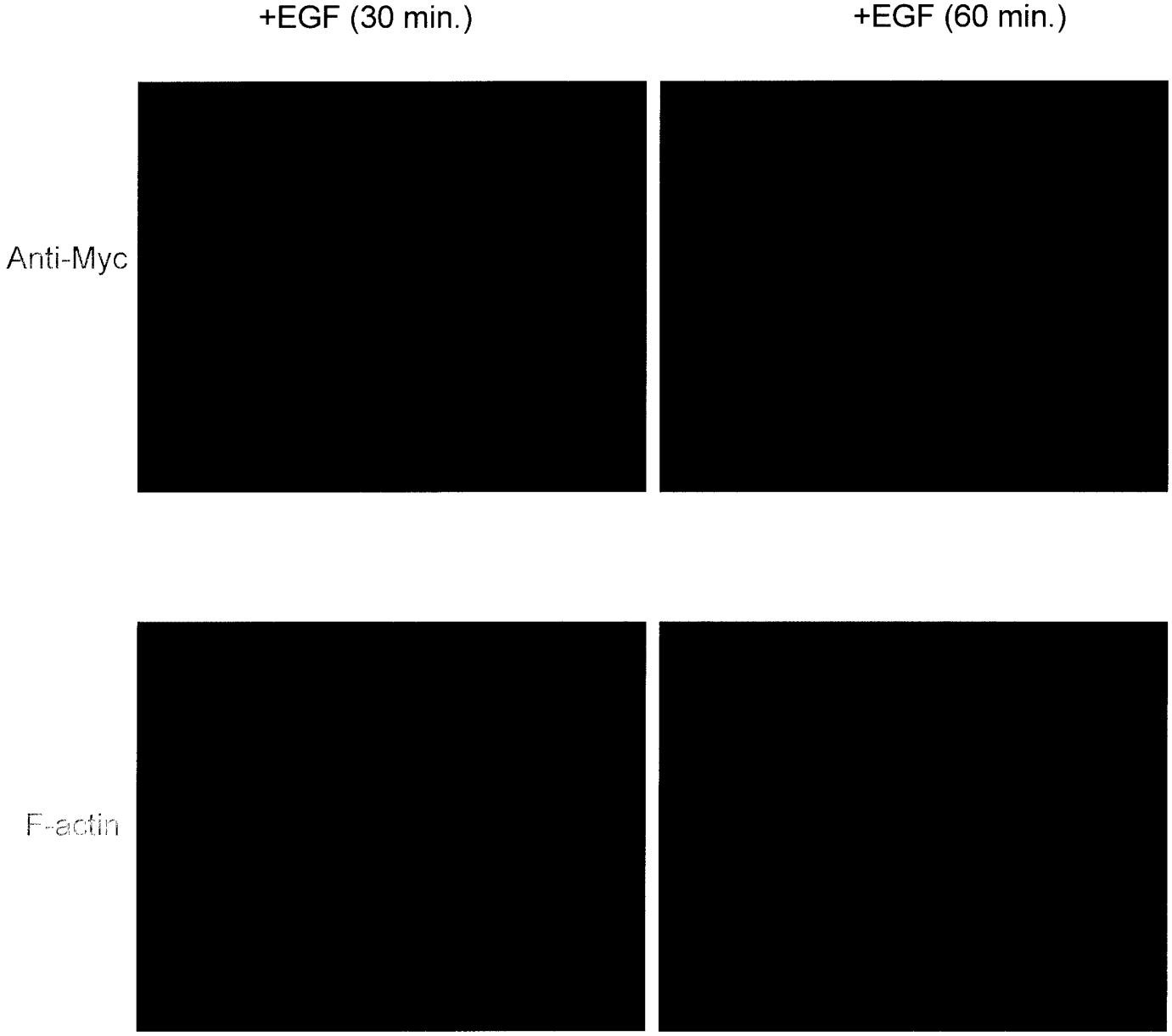
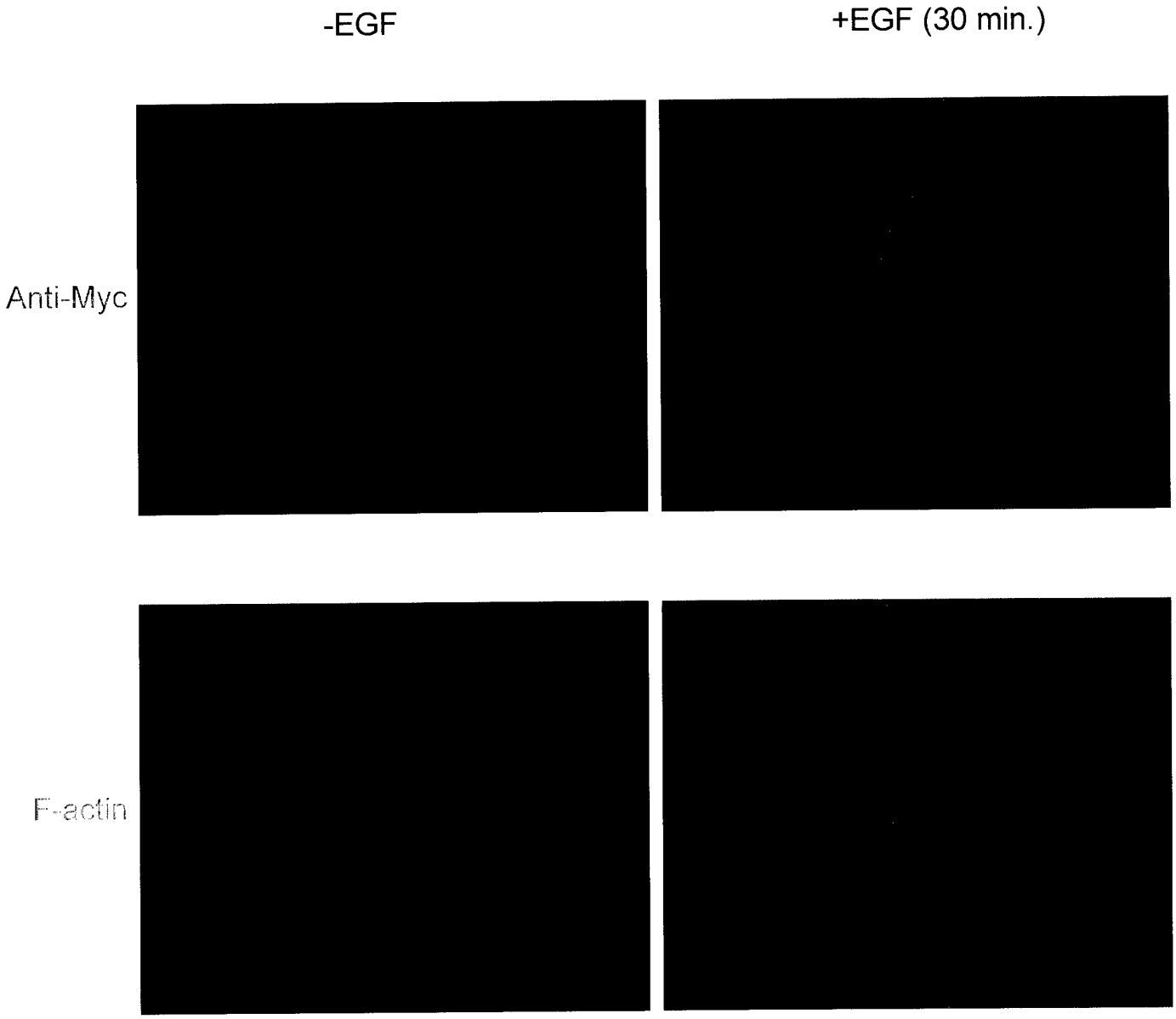


Fig. 9E. FAK-Y397/861F-transfected



Signal Transduction 2002, *1-3*, 1–11

An essential role of ubiquitination in Cbl-mediated negative regulation of the Src-family kinase Fyn

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The Cbl family of ubiquitin ligases function as negative regulators of activated receptor tyrosine kinases by facilitating their ubiquitination and subsequent lysosomal targeting. Here, we have investigated the role of Cbl ubiquitin ligase activity in the negative regulation of a non-receptor tyrosine kinase, the Src-family kinase Fyn. Using mouse embryonic fibroblasts from $Cbl^{+/+}$ and $Cbl^{-/-}$ mice, we demonstrate that endogenous Cbl mediates the ubiquitination of Fyn and dictates the rate of Fyn turnover. By analyzing CHO-TS20 cells with a temperature-sensitive ubiquitin activating enzyme, we demonstrate that intact cellular ubiquitin machinery is required for Cbl-induced degradation of Fyn. Analyses of Cbl mutants, with mutations in or near the RING finger domain, in 293T cells revealed that the ubiquitin ligase activity of Cbl is essential for Cbl-induced degradation of Fyn by the proteasome pathway. Finally, use of a SRE-luciferase reporter demonstrated that Cbl-dependent negative regulation of Fyn function requires the region of Cbl that mediates the ubiquitin ligase activity. Given the conservation of structure between various Src-family kinases and the ability of Cbl to interact with multiple members of this family, Cbl-dependent ubiquitination could serve a general role to negatively regulate activated Src-family kinases.

Keywords: Tyrosine kinase / ubiquitin / regulation / degradation.

Review

Introduction

Src-family kinases (SFKs) constitute a large family of evolutionarily conserved protein tyrosine kinases (PTKs) that mediate crucial biological functions, including critical roles in tissue and organ development, cell differentiation, adhesion and migration, mitogenesis, and immune responses [1, 2]. The ease with which subtle mutations can render SFKs dominantly oncogenic [2] has also made them an important model for understanding the mechanisms of PTK regulation. All SFKs share a conserved domain structure, consisting of a membrane-anchoring N-terminal myristoylation signal, adjacent SH3 and SH2 domains, a kinase domain, and a tyrosine residue near the C-terminal tail whose phosphorylation by the C-terminal Src kinase (CSK) is required for repression [1]. The crystal structures of Src and Hck proteins, together with a large body of mutational data, have established a general model of SFK repression and have suggested potential mechanisms of activation [3, 4]. Intra-molecular SH3 domain binding to a type II polyproline-like helix within the SH2-kinase linker region together with SH2 domain binding to the phosphotyrosine residue near the C-terminus force the kinase domain into an inactive conformation [3, 4]. Activation signals are hypothesized to displace the SH2 and SH3 domains from their intra-molecular ligands, promoting the open, active conformation of the kinase domain and concurrently releasing the SH2 and SH3 domains for assembly of signaling complexes. Consistent with this model, inactivating point mutations in the SFK SH3 or SH2 domains can significantly enhance the kinase activity [5]. Furthermore, mutations within the SH2-kinase linker that abolish its binding to the SH3 domain, or overexpression of high affinity SH3 domain-binding ligands, result in increased kinase activity of Hck, Src, or Lck [6–8]. Similarly, deletion or substitution of the negative regulatory tyrosine within the carboxyl tail of SFKs results in enhanced kinase activity and oncogenesis [2], and deletion of the CSK gene leads to constitutively activated SFKs [9, 10]. Conversely, substitutions that enhance the affinity of the C-terminal phosphotyrosine motif for the SH2 domain decrease the kinase activity [11].

While the above paradigm elegantly accounts for basal repression and provides a plausible scheme for activation of SFKs, it is not clear at present if and how activated SFKs are returned to their basal repressed conformation. Given recent evidence that SFKs require cellular chaperones, such

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1615-4053/2002/0101-0001 $17.50+.50/0

as members of the HSP90 family, for proper folding [12], it is likely that cells utilize additional mechanisms for deactivation of SFKs and, by implication, other PTKs. Without such ancillary mechanisms, activated SFKs could accumulate resulting in deleterious consequences for a cell. Recent studies indicate that the proto-oncoprotein Cbl provides one such mechanism for deactivation of SFKs [13, 14].

Cbl is a member of an evolutionarily conserved family of cytoplasmic proteins that have emerged as negative regulators of PTK signaling [15]. The Cbl homologues in *Caenorhabditis elegans* and *Drosophila* function as negative regulators of epidermal growth factor receptor (EGFR) signaling [16]. Furthermore, genetic ablation of murine Cbl produced hypercellularity and altered development of several organ systems [17, 18], whereas Cbl-b deletion led to hyperproliferation and hyperactivation of immune cells resulting in autoimmunity [19, 20].

Recent studies have demonstrated that Cbl functions as a ubiquitin ligase towards activated receptor tyrosine kinases (RTKs), a modification that facilitates sorting of ligand-activated receptors to lysosomes where they are degraded [21–23]. This proposed mechanism is analogous to the genetically well-characterized, ubiquitin-dependent, lysosomal targeting of yeast membrane receptors [24]. It is thought that lysosomal enzymes degrade the extracellular regions of growth factor receptors, while the cytoplasmic portion of these receptors may be targeted for proteasomal degradation [21–23]. Notably, transfection studies have shown that Cbl can target the activated pools of non-receptor PTKs such as Syk, ZAP-70 and the SFK Fyn for degradation [13, 25, 26]. However, the role of Cbl ubiquitin ligase function in the negative regulation of these non-receptor PTKs has not been addressed. Importantly, if non-receptor PTKs are indeed targeted for Cbl-dependent ubiquitination, their fate is likely to differ from that of ubiquitinated RTKs, as their ubiquitination is likely to target them directly to the proteasome rather than serving as a lysosomal sorting signal.

Defining the role of ubiquitination in Cbl-dependent regulation of SFKs is important not only due to the intrinsic biological significance of SFK regulation, but also because these PTKs interact with Cbl in a manner that is far more complex than the interactions of Cbl with other PTK targets [13]. The evolutionarily conserved N-terminal region tyrosine kinase binding (TKB) domain of Cbl, composed of a four-helical bundle, an EF-hand and an incomplete SH2 domain [27], specifically interacts with negative regulatory phosphorylation sites within Syk/ZAP-70 and EGFR tyrosine kinases, providing a basis for the selective recruitment of Cbl to activated pools of these PTKs [23, 25, 28]. Mutations (in Cbl or its target PTKs) that abrogate Cbl TKB domain interaction with PTKs block Cbl-dependent negative regulation of EGFR, platelet-derived growth factor (PDGFR) and Syk/ZAP-70 PTKs [23, 29, 25, 30, 26]. Furthermore, an intact Cbl RING finger domain, which interacts with E2 ubiquitin conjugating enzymes (UBCs) [31], is also required for ubiquitination and downregulation of the EGFR [21, 32]. Notably, the TKB and RING finger domains, without the C-terminal half of Cbl, are sufficient for the negative regulation of Syk or EGFR, as well as the ubiquitination of EGFR [32, 29, 33].

In contrast to Syk/ZAP-70, which interact with Cbl exclusively *via* its TKB domain, and RTKs, which require a Cbl TKB-mediated interaction for negative regulation, SFK regulation by Cbl is more complex. Previous studies have demonstrated that Cbl-SFK association involves binding between the SFK SH3 domain and the proline-rich sequences in the C-terminal half of Cbl [34]. Furthermore, the SH2 domains of SFKs can interact with phosphopeptide motifs in the C-terminal half of Cbl [35], and an uncharacterized motif in Fyn can interact with the Cbl TKB domain [13]. Consistent with these multiple modes of physical association, a TKB domain mutant of Cbl was fully capable of decreasing the levels and activity of Fyn when analyzed in a 293T cell transfection system; abrogation of Fyn SH3 binding to the proline-rich region of Cbl, in addition to a Cbl TKB mutation, was required to block the effect of Cbl on Fyn [13]. Given these complexities of Cbl-SFK association, and the fact that two of these interactions involve the C-terminal region of Cbl that is dispensable for EGFR and Syk/ZAP-70 regulation, it is critical to determine if Cbl-mediated negative regulation of SFKs indeed involves its activity as a ubiquitin ligase.

Several lines of evidence support the possibility that Cbl-mediated negative regulation of SFKs may be mediated through ubiquitination. We showed that coexpression of Fyn with Cbl resulted in Fyn degradation, and cell lines from $Cbl^{-/-}$ mice showed elevated Fyn levels [13]. Recent studies of other SFKs have revealed them to be targets of ubiquitination [36-39]. For example, Blk was reported to interact with the ubiquitin ligase E6AP and undergo E6AP-dependent ubiquitination and degradation [38]. Similarly, oncogenic *v*-Src as well as *c*-Src, the latter in CSK-deficient fibroblasts, were shown to be ubiquitinated; furthermore, treatment with proteasome inhibitors led to increased protein levels [36, 37]. While the role of the Cbl proteins in the above situations has not been investigated, these findings are consistent with Cbl regulation of SFKs *via* ubiquitination.

Here, we have addressed this hypothesis through analyses of $Cbl^{+/+}$ and $Cbl^{-/-}$ cell lines, Chinese Hamster Ovary (CHO) cells with a temperature-sensitive defect in ubiquitin activating enzyme (E1) and 293T cells co-expressing Cbl and its ubiquitination-deficient mutants. We provide direct evidence that Cbl negatively regulates the SFK Fyn by targeting it for ubiquitination, and that ubiquitination is a critical mechanism to regulate Fyn protein levels and activity. Given the conservation of structure among SFKs, and the ability of Cbl to interact with multiple SFKs, Cbl-dependent ubiquitin-

ation may provide a general mechanism to negatively regulate activated SFKs.

Materials and methods

Cells

293T human embryonic epithelial kidney cells and mouse embryonic fibroblasts (MEFs) from wildtype ($Cbl^{+/+}$) and Cbl knockout ($Cbl^{-/-}$) mice were maintained as previously described [13]. The CHO cell line CHO-TS20, harboring a temperature-sensitive ubiquitin activating enzyme (E1), was maintained as previously described [40].

Antibodies

The following antibodies were used: monoclonal antibody (mAb) 12CA5 (anti-influenza hemagglutinin [HA] epitope tag; IgG2b) [41]; mAb anti-ubiquitin (IgG1, MMS-258R) from *Covance* (Richmond, USA), rabbit polyclonal antibody (pAb) anti-p44/42 MAP kinase (9102) from *New England BioLabs* (Beverly, USA), mAb anti-EGFR (IgG2a, sc-120), pAb anti-Fyn (sc-16) and pAb anti-Cbl (sc-170) from *Santa Cruz Biotechnology* Inc. (Santa Cruz, USA).

Expression plasmids

The HA-ubiquitin, pSRαNeo-CD8-ξ chimera, Cbl and Fyn expression constructs in the pAlterMAX plasmid backbone (*Promega*, Madison, USA) and GFP-Cbl expression constructs in the pCDNA3 vector backbone (*Invitrogen*, Carlsbad, USA) have been previously described [13, 29, 25, 33, 42]. The Cbl RING finger mutant C3AHN was previously referred to as Cbl-C3HC4C5 [33].

Transient Transfections

293T cells were transfected as previously described using the calcium phosphate method. Cell lysates were prepared 48 h post-transfection with Triton X-100 lysis buffer [26] supplemented with 0.1 % sodium dodecyl sulfate (SDS) and 0.1% DOC. TS20 cells were transfected using the *Lipofectamine*™ reagent (*Life Technologies*, Carlsbad, USA), according to the manufacturer's protocol. The cells were cultured at 30°C for 56 h, then either maintained at 30°C (permissive temperature for E1 function) or shifted to 42°C (non-permissive temperature). Cell lysates were prepared in the lysis buffer described above.

Generation of Fyn-overexpressing MEFs

$Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs overexpressing Fyn were established by retrovirus-mediated transfection of $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs. The retroviral construct MSCVpac-Fyn-T was generated by subcloning murine Fyn-T cDNA fragment from pAlterMAX-Fyn into EcoRI digested MSCVpac. Retroviral supernatants were produced and used to infect target cells as described [43]. Bulk transfectant lines were selected in 5 μg/ml puromycin (*Sigma*, St. Louis, USA) and used as such.

Immunoprecipitation, gel electrophoresis and immunoblotting

Immunoprecipitations were performed as described [44]. The immunoprecipitated proteins and total cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidine difluoride (PVDF) membranes (NEN, Boston, USA), and immunoblotted with the indicated antibodies as described [45]. Blots were visualized as described [26]. Photographs were generated by direct scanning of films using a *Hewlett Packard* ScanJet 4c™ scanner, Palo Alto, USA).

Pulse-Chase Analysis of Fyn Protein Turnover

Fyn-overexpressing $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs were grown in 150-mm tissue culture dishes to about 70% confluence, labeled for 1 h at 37°C with 300 μCi/ml EXPRE^{35}S^{35}S labeling mix (NEN), and pulse-chase analysis was performed as previously described [13]. Autoradiography signals were quantified by densitometric analysis of bands using *ScionImage* software (version beta 3b, Frederick, USA).

Luciferase Assay

293T cells were transfected by the calcium phosphate method with a serum response element (SRE)-luciferase reporter construct and the appropriate Cbl and Fyn constructs, as previously described [13]. At 48 h post-transfection, cells were lysed with Cell Culture Lysis Reagent (*Promega*, Madison, USA) and lysate protein concentrations were determined using the *Bradford* assay. Luciferase activity was determined on equal protein aliquots using a Monolight 3010C luminometer (*Analytical Bioluminescence Laboratory* Inc., Sparks, USA and *Luciferin* Reagent (*Promega*).

Results

Severely reduced ubiquitination of Fyn in MEFs derived from Cbl-deficient mice

We have previously demonstrated that Cbl targets the Fyn protein for degradation in a 293T cell overexpression system, and that steady-state levels of Fyn were elevated in $Cbl^{-/-}$ MEF and T cell lines [13]. The latter system provided an opportunity to directly assess if endogenous Cbl controls Fyn ubiquitination. In view of the known difficulties in detecting ubiquitinated proteins such as Fyn [46], we estab-

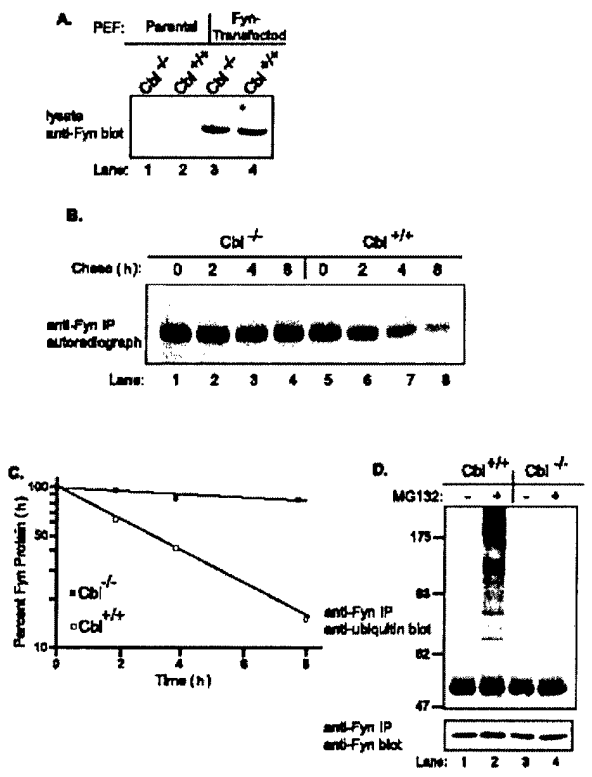

Fig. 1. Stabilization of Fyn protein and impaired Fyn ubiquitination in $Cbl^{-/-}$ primary embryonic fibroblasts.

A. Fyn protein levels in parental *versus* Fyn-transfected primary embryonic fibroblasts (MEFs). Equal amounts (50 μg) of protein lysates from the parental and Fyn-transfected $Cbl^{-/-}$ and $Cbl^{+/+}$ MEFs were resolved by SDS-PAGE and immunoblotted with anti-Fyn antibody.

B. Metabolic pulse-chase analysis of Fyn protein in Fyn-transfected $Cbl^{-/-}$ and $Cbl^{+/+}$ MEFs. $Cbl^{-/-}$ and $Cbl^{+/+}$ MEFs were methionine-starved for 1 h and pulse-labeled with ^{35}S-methionine for 1 h, as described in materials and methods. The cells were incubated in methionine-supplemented, unlabeled medium (chase) for the indicated times (h, hours), and cell lysates were prepared. Anti-Fyn immunoprecipitates (IP) of cell lysates (1 mg) were resolved by SDS-PAGE, and labeled Fyn signals were detected by autoradiography.

C, the radioactive Fyn signals in **B** were quantified using densitometry, expressed as a percentage of the maximal signal intensity and plotted as a function of chase times.

D, impaired Fyn ubiquitination in $Cbl^{-/-}$ MEFs. Fyn-transfected $Cbl^{-/-}$ and $Cbl^{+/+}$ MEFs were incubated with 50 μM MG132 (+) or DMSO control (−) for 5 h and then lysed. Anti-Fyn immunoprecipitates of 1 mg aliquots of lysate were immunoblotted with anti-ubiquitin antibody (top panel), followed by anti-Fyn antibody (bottom panel).

lished a matched pair of $Cbl^{+/+}$ and $Cbl^{-/-}$ MEF lines expressing approximately ten-fold higher levels of Fyn compared to parental MEFs (Fig. 1A).

To determine the impact of the presence or absence of endogenous Cbl on the stability of Fyn protein, we carried out a metabolic pulse-chase analysis of Fyn in the Fyn-transfected $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs. Equal aliquots of cell lysates were subjected to anti-Fyn immunoprecipitation and radiolabeled Fyn was detected by autoradiography (Fig. 1B). Comparable ^{35}S-Fyn signals were observed in the two cell lines prior to chase (time zero) (Fig. 1B, compare lane 1 with lane 5). Whereas the radiolabeled Fyn signal in $Cbl^{+/+}$ MEFs showed a substantial time-dependent reduction of nearly 80% over the chase period, with a half-life of about 3 h, Fyn protein in $Cbl^{-/-}$ cells was substantially more stable with only a small decrease in signal during the chase period (Fig. 1B and C). These results established that endogenous Cbl controls the stability of the Fyn protein, and provided crucial reagents to directly assess if Cbl regulates the ubiquitination of Fyn.

To assess Fyn ubiquitination in Fyn-transfected $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs, the cells were incubated for 5 h with (+) or without (−) the proteasome inhibitor MG132, and their lysates were subjected to anti-Fyn immunoprecipitations followed by anti-ubiquitin immunoblotting. A low but detectable ubiquitin signal, seen as a smear similar to ubiquitinated species of other SFKs [37, 39], was observed in anti-Fyn immunoprecipitates of $Cbl^{+/+}$ MEFs incubated without MG132; this signal dramatically increased upon MG132 treatment (Fig. 1D, top panel, compare lane 1 with lane 2). In contrast, the ubiquitin signal was essentially undetectable in anti-Fyn immunoprecipitates of $Cbl^{-/-}$ MEFs and remained very low even after MG132 treatment (Fig. 1D, top panel, compare lane 2 with lane 4). Anti-Fyn immunoblotting showed that MG132 treatment increased the Fyn protein level in $Cbl^{+/+}$ but not $Cbl^{-/-}$ MEFs (Fig. 1D, bottom panel). These findings demonstrate that Fyn protein undergoes ubiquitination, and that the level of endogenous Cbl protein controls the extent of Fyn ubiquitination.

Intact cellular ubiquitination machinery is essential for Cbl-mediated Fyn degradation

Stabilization of Fyn protein in $Cbl^{-/-}$ cells, together with accumulation of ubiquitinated Fyn in MG132-treated $Cbl^{+/+}$ cells, strongly suggested that Cbl-induced ubiquitination serves as a signal for proteasome-mediated degradation. To directly assess the requirement of Fyn ubiquitination for its Cbl-induced degradation, we utilized CHO-TS20 cells. In these cells, the ubiquitin activating enzyme (E1) is fully active at 30°C but nonfunctional at 42°C [40], allowing manipulation of Fyn ubiquitination by using a temperature shift.

Very little Fyn ubiquitination (anti-Fyn immunoprecipitations immunoblotted with anti-ubiquitin) was observed in the ab-

sence of co-transfected Cbl at either temperature (Fig. 2A, lanes 1-5). In contrast, co-expression of Cbl resulted in a marked increase in the levels of ubiquitinated Fyn when cells were maintained at 30°C (Fig. 2A, lane 6). When these cells were shifted to 42°C, Fyn ubiquitination decreased rapidly with nearly undetectable signals after 9 h (Fig. 2A, compare lane 6 with lanes 9-10). Thus, the level of Cbl-induced ubiquitination of Fyn could be precisely regulated in CHO-TS20 cells upon temperature shift.

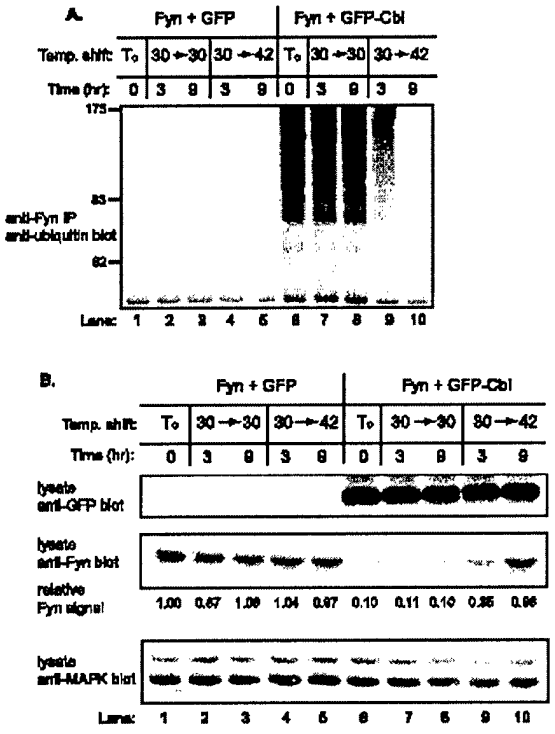

Fig. 2. Cbl-mediated loss of Fyn protein requires intact cellular ubiquitination machinery.

A. impaired Fyn ubiquitination upon E1 inactivation in Cbl-transfected CHO-TS20 cells. CHO-TS20 cells were transfected with Fyn (0.2 μg) expression plasmid together with 4 μg of GFP or GFP-Cbl plasmids and incubated at 30°C for 56 h. At this point (T_o), cells were either maintained at 30°C (30 → 30) or shifted to 42°C (30 → 42) for the indicated times. Anti-Fyn immunoprecipitates from aliquots of lysate protein (1 mg) were immunoblotted with antiubiquitin antibody.

B. stabilization of Fyn protein upon E1 inactivation in Cbl-transfected CHO-TS20 cells. Equal amounts (30 μg) of the same cell lysate used in **A** were immunoblotted with anti-GFP antibody (top panel), anti-Fyn antibody (middle panel), and anti p42/44 MAPK antibody (bottom panel). The levels of Fyn protein were quantified by densitometry, and the values at various times are expressed as a function of the initial Fyn protein level (lane 1) that was assigned a value of 1.0.

The cell lysates used above were directly immunoblotted with anti-GFP and anti-Fyn antibodies to assess the levels of transfected GFP-Cbl and Fyn proteins, respectively. As anticipated, cells co-transfected with GFP-Cbl and Fyn showed a marked reduction in Fyn protein levels when compared to cells cotransfected with GFP vector (Fig. 2B, middle panel, compare lane 1 with lane 6; densitometric units of 1.0 *versus* 0.1). When transfected cells were maintained at the permissive temperature (30°C), no substantial changes in the steady-state levels of Fyn protein were observed. In contrast, when Fyn plus GFP-Cbl transfected cells were shifted to 42°C, a marked time-dependent increase in Fyn protein levels was observed (Fig. 2B, middle panel, lanes 6–10; densitometric units of 0.35 and 0.96 at 3 h and 9 h at 42°C *versus* 0.11 and 0.10 at 30°C, respectively). Relatively little change in Fyn protein level was observed when GFP and Fyn transfected cells were shifted to 42°C (Fig. 2B, middle panel, lanes1–5; densitometric units of 1.04 and 0.97 at 3 h and 9 h at 42°C *versus* 0.87 and 1.09 at 30°C, respectively). Anti-MAP kinase immunoblotting of cell lysates revealed no substantial changes in the levels of MAP kinase protein (Fig. 2B, bottom panel). These results establish that Cbl-mediated degradation of Fyn requires intact cellular ubiquitination machinery.

Cbl-mediated ubiquitination and degradation of Fyn requires an intact Cbl RING finger domain and Fyn SH3 domain

Given the ability of Cbl to control Fyn ubiquitination, we wished to determine if this activity is mediated by the Cbl RING finger-domain encoded ubiquitin ligase activity. For this purpose, we compared the ability of wildtype Cbl protein with its RING finger domain mutants to target Fyn for ubiquitination. To assess Cbl-dependent Fyn ubiquitination *in vivo*, 293T cells were co-transfected with Fyn together with GFP or GFP-Cbl, and a plasmid encoding HA-tagged ubiquitin to facilitate detection of ubiquitinated Fyn.

As expected [32], transfection with GFP-Cbl led to easily detectable ubiquitination of EGFR and this signal was markedly enhanced by MG132 treatment of cells (Fig. 3A, top panel, compare lane 9 with lane 10). Relatively little ubiquitin signal was observed on Fyn in the absence of co-transfected Cbl. In contrast, co-expression of GFP-Cbl led to easily detectable ubiquitination of Fyn, which was accompanied by an expected decrease in the level of Fyn protein (Fig. 3A, compare lane 1 with lane 3). MG132 treatment of cells prior to lysis resulted in marked accumulation of ubiquitinated Fyn and an increase in the level of Fyn protein (Fig. 3A, compare lane 3 with lane 4). Equivalent expression of GFP-tagged

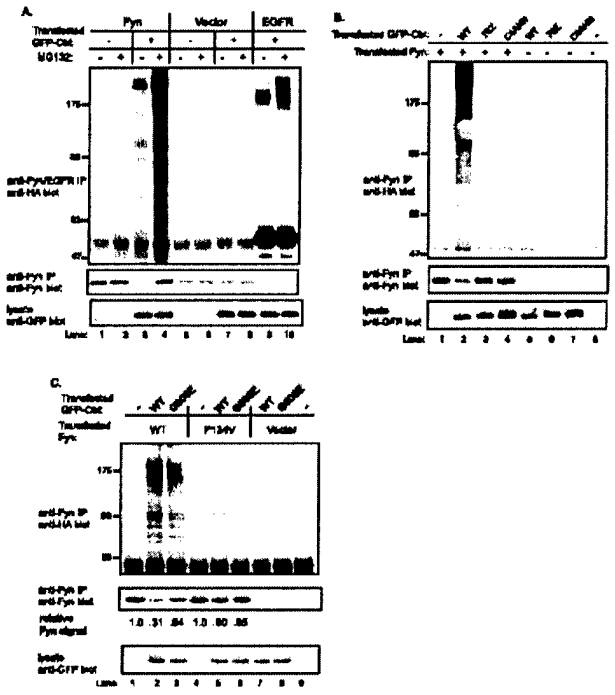

Fig. 3. Cbl-dependent ubiquitination of Fyn in 293T cells and an essential role for the Cbl RING finger domain.

A. Cbl-dependent Fyn ubiquitination is enhanced by treatment with a proteasome inhibitor. 293T cells were transfected with plasmids encoding HA-ubiquitin (7 μg), Fyn (0.15 μg), EGFR (0.15 μg), GFP-Cbl (+) (3 μg) or a GFP (−) control (3 μg). 5 h prior to cell lysate preparation, cells were treated with 50 μM MG132 (+) or DMSO control (−). Anti-Fyn or anti-EGFR immunoprecipitates from aliquots of lysate protein (800 μg) were immunoblotted with anti-HA antibody (top panel) followed by anti-Fyn antibody (middle panel). Equal aliquots (30 μg) of the same cell lysates used above were immunoblotted with anti-GFP antibody (bottom panel).

B. An intact RING finger domain is required for Cbl-dependent Fyn ubiquitination. 293T cells were transfected with the indicated expression plasmids, lysed and anti-Fyn immunoprecipitations were carried out as in **A** and immunoblotted with anti-HA antibody (top panel) and with anti-Fyn antibody (middle panel). The lysate proteins (30 μg) were immunoblotted with anti-GFP antibody (bottom panel).

C. The role of Fyn SH3 domain and Cbl TKB domain-mediated interaction for Cbl-dependent Fyn ubiquitination. 293T cells were transfected with the vector, wildtype Fyn or Fyn-SH3 domain mutant (P134V), lysed and anti-Fyn immunoprecipitations were carried out as in **A** and immunoblotted with anti-HA antibody (top panel) and with anti-Fyn antibody (middle panel). The lysate proteins (10 μg) were immunoblotted with anti-GFP antibody (bottom panel). The levels of Fyn protein were quantified by densitometry, and the values are expressed as a function of the initial Fyn protein level for each Fyn construct (lane 1 and 4) that was assigned a value of 1.0.

Cbl protein in the appropriate lysates was confirmed by anti-GFP immunoblotting of whole cell lysates (Fig. 3A, bottom panel).

Next, we examined if the RING finger domain is required for Cbl-mediated ubiquitination of Fyn. The Cbl mutant C3AHN contains four point mutations predicted to abrogate coordination of both zinc atoms that stabilize the RING finger domain [33], whereas the naturally occurring Cbl-70Z mutant, which is unable to induce Fyn degradation [13], has a deletion of the critical linker region that provides additional essential contacts for UBC binding [31]. In contrast to wildtype Cbl, both 70Z and C3AHN Cbl RING finger mutants were unable to mediate Fyn ubiquitination (Fig. 3B, top panel) despite their equivalent or higher expression levels compared to wildtype Cbl (Fig. 3B, bottom panel). Thus, the RING finger domain-mediated ubiquitin ligase activity of Cbl is necessary for Cbl-dependent ubiquitination of Fyn.

Previous studies have demonstrated that the Fyn SH3 domain provides the primary mode of association with Cbl by interacting with its proline-rich region [13]. Furthermore, mutation of the Cbl TKB domain alone had no significant effect on Cbl-mediated Fyn degradation. However, the Cbl TKB domain was capable of associating with Fyn [13], and recent studies using Src have suggested that the Cbl TKB domain binds to the SFK activation loop phosphorylation site [47], which is conserved among all SFKs. In order to extend our structure-function analyses and establish which domains of Cbl and Fyn are required for Fyn ubiquitination, we tested the ability of a Fyn SH3 domain mutant (P134V) [13] to be ubiquitinated by wildtype Cbl or its TKB domain mutant (G306E). Compared to wildtype Cbl, the TKB domain mutant was still able to mediate Fyn ubiquitination and degradation although to a lesser extent (Fig. 3C, top and middle panel, compare lanes 1–3) when expressed at a level equivalent to wildtype Cbl (Fig. 3C, bottom panel). In contrast, the Fyn SH3 domain mutant showed only minor ubiquitination and degradation when coexpressed with wildtype Cbl. Essentially no ubiquitination or degradation of the Fyn SH3 mutant was observed when coexpressed with the Cbl G306E mutant, and Fyn protein levels were unchanged (Fig. 3C, middle panel). Thus, the Fyn SH3 domain mediates the predominant physical interaction required for Cbl-mediated Fyn ubiquitination, while the Cbl TKB domain appears to mediate a less dominant mode of interaction.

The RING finger domain plays an essential role in Cbl-mediated negative regulation of Fyn-dependent cellular activation

In order to determine the effect of Cbl-dependent ubiquitination on Fyn-mediated cellular activation, we compared the effects of wildtype Cbl and its RING finger domain mutants on Fyn kinase-dependent transactivation of the serum re-

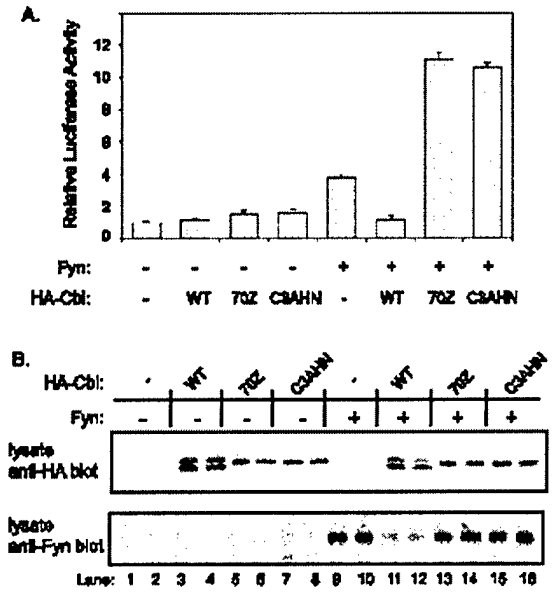

Fig. 4. The RING finger domain is required for Cbl-dependent negative regulation of Fyn-induced transcriptional activation of a SRE-luciferase reporter.

A. mutations in the RING finger domain of Cbl blocks the negative regulation of SRE-luciferase activation. 293T cells were transfected with plasmids encoding the SRE-luciferase reporter (5 μg), CD8-ζ(0.5 μg) and the indicated combinations of Fyn (0.1 μg), HA-Cbl, HA-Cbl-70Z and HA-Cbl-C3AHN (1 μg) or pAlterMAX vector (−). Cells were lysed 48 h after transfection and equal aliquots of lysate protein were used to assay the luciferase activity. The luciferase activity was expressed relative to the activity of lysates transfected with the reporter in the absence of Fyn or Cbl. Results represent the mean +/− one standard deviation of five replicate transfections.

B. analysis of Fyn protein levels in transfected cells used for SRE-luciferase assay. Aliquots of lysate protein (10 μg) from 2 of the 5 replicate samples analyzed in **A** were resolved by SDS-PAGE and immunoblotted with anti-HA (top panel) and anti-Fyn (bottom panel) antibodies.

sponse element (SRE) linked to a luciferase reporter [48]. 293T cells were transfected with the SRE-luciferase reporter plasmid and either Fyn alone or Fyn in combination with wildtype Cbl or its RING finger domain mutants. As expected [13], the expression of Fyn protein led to a modest increase in SRE-luciferase activity compared to mock-transfected cells (Fig. 4A), and this increase was suppressed upon co-expression of wild-type Cbl. In contrast, co-expression of the Cbl RING finger mutant C3AHN as well as the 70Z mutant [13] resulted in a marked enhancement of Fyn-dependent SRE-luciferase reporter activity (Fig. 4A). Expression of Cbl proteins without Fyn had no effect on the SRE luciferase activity. Analysis of cell lysates demonstrated the expected effects of Cbl proteins on Fyn protein levels and confirmed the equivalent expression of various Cbl constructs (Fig. 4B). Overall, these data demonstrate that the RING finger domain, which is required for Fyn ubiquitination and degradation, is also critical for functional negative regulation of Fyn by Cbl.

Discussion

The recently identified function of Cbl as a ubiquitin ligase [21, 32] and our earlier results that Cbl functions as a negative regulator of SFKs [13] led us to hypothesize that Cbl ubiquitin ligase activity provides a physiological mechanism to control the levels of activated SFKs. Here we provide several lines of evidence in support of this hypothesis by examining the regulation of SFK Fyn.

Analyses of multiple cell types, including mouse embryonic fibroblasts, CHO-TS20 cells and 293T human embryonic kidney cells provide evidence for Cbl-dependent ubiquitination of Fyn. An accumulation of ubiquitinated Fyn was also observed upon MG132 treatment of a Jurkat T cell line stably overexpressing Cbl [26] (*Navin Rao, Hamid Band*, unpublished results). Importantly, we show that lack of endogenous Cbl leads to a drastic deficiency in Fyn ubiquitination in $Cbl^{-/-}$ MEFs. The reduction in Fyn ubiquitination in $Cbl^{-/-}$ cells is accompanied by a substantial increase in endogenous Fyn levels [13] and a marked increase in the half-life of the Fyn protein, indicating that Cbl-dependent ubiquitination is a critical determinant of Fyn turnover. It is notable that there are two other mammalian Cbl family members [15]. Whether drastically reduced Fyn ubiquitination in $Cbl^{-/-}$ MEFs reflects a lack of expression of other Cbl family members or a lesser role for these proteins in Fyn ubiquitination will require further investigation.

A complimentary line of evidence for a critical role of Cbl-dependent ubiquitination in regulating Fyn protein levels was provided by analyses of CHO-TS20 cells, which express a thermolabile ubiquitin activating (E1) enzyme. This genetic approach provided further evidence that ubiquitin machinery is essential for Cbl to induce the degradation of Fyn. The results obtained in MEFs and CHO-TS20 cells clearly implicate the ubiquitin ligase activity of Cbl in the negative regulation of Fyn. *In vivo* analysis in 293T cells, using Cbl RING finger domain mutants, established that this indeed was the case. Taken together, our results establish Cbl-dependent ubiquitination as an important mechanism of negative regulation for Fyn, a prototype SFK. Given the ability of Cbl to interact with multiple SFKs and the conservation of structure among members of the SFK family, we propose that Cbl-dependent ubiquitination may provide a general mechanism to negatively regulate activated SFKs. The relatively intense

ubiquitin signal on higher molecular weight species, as compared to Fyn signal, is likely due to multi-ubiquitinated Fyn providing increased numbers of epitopes reactive with anti-ubiquitin antibody. We consider it unlikely that these higher molecular weight species represent a Fyn-associated protein, as lysates were prepared in lysis buffer containing SDS and deoxycholate in order to disrupt protein-protein interactions. Indeed, under such conditions, Fyn-Cbl association was disrupted (data not shown). Moreover, these experiments were performed under optimized conditions to detect Cbl-mediated Fyn ubiquitination and degradation. –

Demonstration of a non-receptor PTK as a target of Cbl-mediated ubiquitination is of considerable significance since all of the targets identified previously are RTKs. Ubiquitin modification of RTKs facilitates sorting to lysosomes for degradation, thus accounting for receptor downregulation. In contrast, ubiquitination of non-receptor PTKs, such as Fyn, is likely to serve as a direct targeting signal for proteasomal degradation, as supported by our results using proteasome inhibitors. While *in vivo* studies and the known direct association between Cbl and SFKs are consistent with Cbl-mediated ubiquitination of SFKs, further analyses using purified SFK, Cbl and ubiquitination enzymes in *in vitro* reconstitution assays will be needed to establish this definitively.

It is likely that Cbl-mediated degradation functions in concert with other mechanisms for deactivation of SFKs, such as the return of activated SFKs to their repressed state through CSK-mediated phosphorylation of the C-terminal tyrosine and potential chaperone-mediated folding into a closed, inactive conformation. The ability of Cbl to target SFKs for ubiquitination and degradation also provides a likely explanation for why Cbl, unlike other SH3 domain ligands such as Sin and HIV NEF [48, 6, 8], does not activate SFKs. This proposal is supported by the ability of ubiquitin ligase-deficient Cbl mutants, such as 70Z and C3AHN, to activate rather than downregulate SFK activity.

The proposed role of ubiquitin in Cbl-mediated SFK regulation is consistent with recent findings that other SFKs, such as v-Src, *c*-Src, Lyn and Blk undergo ubiquitination [36–39]. A recent report published while the present paper was under review indicates that Cbl can indeed function as a ubiquitin ligase towards v-Src and *c*-Src [49]. Interestingly, Blk was shown to interact with and serve as a target of the HECT domain-containing ubiquitin ligase E6AP, which has been previously implicated in ubiquitin-dependent degradation of the nuclear tumor suppressor protein p53 by the human papilloma-virus oncoprotein E6 [50]. Whether E6AP is a physiological ubiquitin ligase for Blk or other SFKs, and whether Cbl and E6AP might work in concert are obvious questions that will require further examination.

A number of observations suggest that Cbl-dependent ubiquitination and degradation primarily target the activated pool of SFKs. The SFK SH3 and SH2 domains, which are primarily responsible for association with Cbl [13], are predicted to be intra-molecularly sequestered in repressed SFKs but available for inter-molecular interactions after activation. The additional interaction between Cbl and Fyn, mediated *via* Cbl's TKB domain, is also likely to involve an activation-dependent autophosphorylation site on Fyn, very likely the activation loop phosphorylation site [47]. Consistent with our proposal, mutation of the Fyn SH3 domain drastically reduced Cbl-mediated Fyn ubiquitination and degradation. However, mutation of the Cbl TKB domain also reduced its ability to induce Fyn ubiquitination and degradation quite significantly. Together, these results suggest that both the Fyn SH3 domain and Cbl-TKB domain-mediated interactions, expected only upon activation of Fyn, contribute to Cbl-dependent Fyn ubiquitination. The more dominant effect of abrogating the Fyn SH3 domain-mediated interaction with Cbl may reflect a requirement for this primary association in order for the secondary Cbl TKB-domain-mediated Cbl-Fyn interaction to occur. Further support for selective regulation of the activated pool of SFKs by Cbl is provided by the observation that the level of autophosphorylated Fyn was markedly increased in $Cbl^{-/-}$ MEFs and T cell lines when compared to their $Cbl^{+/+}$ counterparts [13]. We also show here that wild-type Cbl reduces whereas ubiquitination-defective Cbl mutants increase the Fyn-dependent SRE luciferase reporter activity, a readout of the kinase activity of SFKs. Notably, co-expression of Cbl was also shown to reduce the Src-dependent induction of DNA synthesis in NIH 3T3 cells, and the inhibitory effect of Cbl was abrogated by deletion of the RING finger domain [14]. Finally, ubiquitination of Src and Blk also correlated with their kinase activity [36–38], and CSK-deficient cells were shown to have elevated kinase activity but reduced protein levels of Src, Fyn and Lyn [9, 10]. It will be important to determine if reduction in SFK protein levels in these situations is Cbl-dependent.

While our results support a model that the major function of Cbl is to downregulate the level of activated SFKs by ubiquitin-mediated degradation, other studies have suggested that Cbl transduces signals downstream of SFKs. For example, several SFK-mediated cellular functions, such as integrin-induced macrophage spreading and bone resorption by osteoclasts, were severely reduced when cells were treated with Cbl antisense oligonucleotides [51, 52]. Furthermore, introduction of Cbl into v-abl transformed NIH 3T3 cells restored cell adhesion [43, 53], and a truncated Cbl protein (Cbl 1–480) lacking the C-terminal region, enhanced lamellipodia formation in transfected NIH 3T3 cells [54]. It is therefore possible that Cbl can downregulate SFKs by targeting them for ubiquitination while simultaneously serving as an adapter for SH2 domain-containing proteins, thereby positively regulating signal transduction. In this regard, it is notable that the C-terminal phosphorylation sites of Cbl interact with the p85 subunit of PI3 kinase, the Rac/Rho exchange factor Vav and Crk adapter proteins [15], all of which are

known to be involved in cytoskeletal remodeling, cell spreading and cell migration.

In conclusion, our results demonstrate that Cbl functions as a key regulator of the SFK Fyn by enhancing its ubiquitination and subsequent degradation *via* the proteasome. The negative regulatory role of Cbl is dependent on intact cellular ubiquitin machinery as well as the Cbl RING finger domain, which recruits the ubiquitin machinery. Given the ability of Cbl to interact with multiple SFKs and the conservation of structure among various SFKs, we propose that Cbl-dependent ubiquitination may provide a general mechanism to negatively regulate activated SFKs.

Acknowledgments

We thank Dr. *Ger Strous (University Medical Center Utrecht*, Netherlands) for providing the CHO-TS20 cell line, Dr. *Dirk Bohmann (EMBO*, Heidelberg, Germany) for the HA-ubiquitin expression construct, Dr. *Alexander Tysgankov (Temple University*, Philadelphia, USA) for the pHIT60 and pMD.G packaging plasmids and Dr. *Robert Hawley (University of Toronto*, Toronto, Canada) for the MSCVpac retroviral vector.

This work was supported by grants to Hamid Band from the *National Institutes of Health* (CA87986, CA75075 and CA76118) and the *American Cancer Society* (066-04-CIM). *Navin Rao* is a *Howard Hughes Medical Institute* Predoctoral Fellow. *Christopher E. Andoniou, Amiya Ghosh* and *Patrice Douillard* are US Department of Defense Breast Cancer Research Program Postdoctoral Fellows (*C. E. Andoniou* and *A. Ghosh* by DAMD 17-98-1-8032 and *P. Douillard* by DAMD17-99-1-9085).

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[Received: July 10, 2001; accepted: November 7, 2001]

Negative regulation of Lck by Cbl ubiquitin ligase

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Communicated by K. Frank Austen, Harvard Medical School, Boston, MA, January 31, 2002 (received for review November 13, 2001)

The Cbl-family ubiquitin ligases function as negative regulators of activated receptor tyrosine kinases by facilitating their ubiquitination and subsequent targeting to lysosomes. Cbl associates with the lymphoid-restricted nonreceptor tyrosine kinase Lck, but the functional relevance of this interaction remains unknown. Here, we demonstrate that T cell receptor and CD4 coligation on human T cells results in enhanced association between Cbl and Lck, together with Lck ubiquitination and degradation. A $Cbl^{-/-}$ T cell line showed a marked deficiency in Lck ubiquitination and increased levels of kinase-active Lck. Coexpression in 293T cells demonstrated that Lck kinase activity and Cbl ubiquitin ligase activity were essential for Lck ubiquitination and negative regulation of Lck-dependent serum response element-luciferase reporter activity. The Lck SH3 domain was pivotal for Cbl-Lck association and Cbl-mediated Lck degradation, with a smaller role for interactions mediated by the Cbl tyrosine kinase-binding domain. Finally, analysis of a ZAP-70-deficient T cell line revealed that Cbl inhibited Lck-dependent mitogen-activated protein kinase activation, and an intact Cbl RING finger domain was required for this functional effect. Our results demonstrate a direct, ubiquitination-dependent, negative regulatory role of Cbl for Lck in T cells, independent of Cbl-mediated regulation of ZAP-70.

Protein tyrosine kinase (PTK) activation is an early and necessary event for cellular activation upon engagement of antigen receptors such as the B and T cell receptors (TCR) and Fc receptors (1). The initial event involves the activation of membrane-anchored Src-family kinases (SFKs), such as Lck and Fyn, which phosphorylate the immunoreceptor tyrosine-based activation motifs within the signaling subunits of the receptor, thus creating docking sites to recruit Syk or ZAP-70 PTKs. Genetic and biochemical analyses have established that serial SFK and Syk/ZAP-70 activation is required for antigen receptor signaling (1).

Lck plays a particularly important role in the immune system, and this lymphoid-restricted SFK plays a vital role in T cell development and function (2). Although accentuation of the $CD4^-CD8^-$ T cell developmental block in $Lck^{-/-}$ mice by concurrent Fyn-deficiency suggests partial redundancy, Fyn does not restore peripheral T cell activation in $Lck^{-/-}$ mice, thus indicating an essential, nonredundant role of Lck in T cell activation (3–5).

Lck activation by *Herpes saimari* tyrosine kinase-interacting protein (TIP)-transforming protein and mutational analysis of Lck and other SFKs has established that their unregulated activity results in oncogenicity (6, 7). Thus, precise regulation of Lck is vital for physiological function. Intramolecular SH2 domain-binding to the negative regulatory phosphotyrosine residue near the C terminus, and the SH3 domain-binding to the SH2-kinase linker region maintains SFKs in an inactive, closed conformation, accounting for their basal repressed state. On cellular activation, these intramolecular interactions cease resulting in derepression of the kinase domain while concurrently promoting SH2 and SH3 domain-mediated protein–protein interactions that are essential for signal transmission (8).

In contrast to mechanisms of basal repression and activation that are well supported by crystal structural studies (8), mechanisms of SFK inactivation have been less clear. Tyrosine phosphatases, such as SHP-1, provide one likely mechanism (9); however, it is unclear whether dephosphorylation is sufficient to revert activated Lck back into its inactive state, a process that would also require C-terminal Src kinase (CSK)-mediated phosphorylation of Lck and possibly cellular chaperones such as Hsp90 (10). Recent studies indicate that the Cbl protein family provides a new mode of negatively regulating the activated pools of SFKs (11, 12).

With three distinct mammalian members, the Cbl family of multidomain-signaling proteins is highly conserved in sequence and domain architecture from *Caenorhabditis elegans* to man (13, 14). The conserved N-terminal tyrosine kinase-binding (TKB) domain binds to activation-induced phosphotyrosine motifs and the linker helix and a RING finger domain mediate physical interaction with the E2 ubiquitin (Ub) conjugating enzymes of the Ub pathway (15). Thus Cbl can function as an E3 Ub ligase toward activated PTKs bound to the Cbl TKB domain (15). Cbl-mediated ubiquitination of activated receptor tyrosine kinases serves as a lysosomal targeting signal (16), whereas ubiquitination of nonreceptor PTKs Syk and ZAP-70 targets them for proteasomal degradation (17, 18).

Several SFKs, such as Fyn, Src, Lck ,and Lyn, interact with Cbl by way of the SFK SH3 domain binding to the Cbl proline-rich region, and possibly by way of the SFK SH2 domain binding to phosphorylated Cbl (13). Recent results have shown that Cbl can dramatically reduce the pool of active Fyn through enhanced degradation (11), suggesting a role for Cbl in negatively regulating SFKs. However, analyses of Src-dependent cell spreading and migration in macrophages (19) and bone resorption in osteoclasts (20) have suggested a positive role of Cbl in these responses downstream of Src. Therefore, it is unclear whether negative regulation of Fyn by Cbl is a specialized case or generalizable to other SFKs such as Lck. This question is of obvious importance because the vast majority of cellular Lck is anchored to the plasma membrane, whereas Fyn and Src localize primarily to intracellular vesicles (21). Furthermore, only Lck directly associates with T cell coreceptors CD4/CD8. Here, we demonstrate that Cbl and Lck associate upon TCR/CD4 activation and Lck is ubiquitinated and degraded by the proteasome. These studies support a novel role for Cbl-dependent ubiquitination and degradation in the negative regulation of Lck. Together with previous results, using Fyn, this study suggests a general role for Cbl to regulate SFKs.

Materials and Methods

Cells. The 293T human embryonic kidney epithelial cells, T cell lines 230 and 206 from $Cbl^{+/+}$ and $Cbl^{-/-}$ mice, the human $CD4^+$ T cell clone SPF1, and the ZAP-70-deficient Jurkat T cell

Abbreviations: IP, immunoprecipitation; PTK, protein tyrosine kinase; SFK, Src-family kinase; SRE, serum response element; TCR, T cell receptor; TKB, tyrosine kinase binding; Ub, ubiquitin; WT, wild type; HA, hemagglutinin; MAPK, mitogen-activated protein kinase.

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www.pnas.org/cgi/doi/10.1073/pnas.062055999

line expressing the SV40 T antigen (p116-T) were all maintained as described (11, 18, 22).

Antibodies. The antibodies used were: monoclonal antibody (mAb) 12CA5 [anti-influenza hemagglutinin (HA)]; mAb 4G10 (anti-pTyr); mAb SPV-T3b (anti-CD3ε); mAb OKT4 (anti-CD4); mAb W6/32 (anti-MHC I); mAb anti-Ub from Covance; rabbit polyclonal antibody anti-p44/42 mitogen-activated protein kinase (MAPK), polyclonal antibody anti-p44/42 phospho-MAPK, and polyclonal antibody anti-phospho-Src from Cell Signaling Technology (Beverly, MA) and mAb anti-Lck and polyclonal antibody anti-Cbl from Santa Cruz Biotechnology.

Expression Plasmids. The Cbl expression constructs in pAlterMAX and pCDNA3 vector backbone have been described (11, 17, 23). To generate pAlterMAX-Lck constructs, pDKCR Lck and mutant constructs (18) were used as templates for PCR followed by cloning into the pAlterMAX vector. The Lck SH2 (R154K), SH3 (W97A), and double mutant were generated by using Quickchange Mutagenesis (Invitrogen). The plasmid encoding HA-Ub was kindly provided by D. Bohmann (European Molecular Biology Organization, Heidelberg, Germany).

Cell Lysis. Cell lysates were prepared in one of the following buffers as indicated in the figure legends: Triton lysis buffer (11); RIPA buffer (0.15 M NaCl/0.05 M Tris, pH 7.5/1% Triton X-100/1% sodium deoxycholate/0.1% SDS), Triton lysis buffer containing 0.1% SDS and 0.5% deoxycholate; and SDS lysis buffer, Triton lysis buffer containing 1% SDS.

Transient Expression. The 293T cells were transfected by using the calcium phosphate method, and p116T cells were transfected by electroporation (11). Cell lysates were prepared 48 h posttransfection.

T Cell Stimulation. The p116-T cells were stimulated through the TCR by adding SPV-T3b antibody for the indicated times, and cells were lysed. SPF1 T cells were washed in RPMI medium 1640 containing Hepes and glutamine, resuspended at 50×10^6/ml, and incubated with the following mAb (ascites 1:100) SPV-T3b, OKT4, or W6/32 (anti-MHC I) control. After incubation on ice for 30 min, the cells were washed, warmed to 37°C, and bound antibodies were cross-linked by using rabbit anti-mouse antibody.

Immunoprecipitation, Gel Electrophoresis, and Immunoblotting. Immunoprecipitations (IPs) were performed as described (17). The immunoprecipitated proteins and total cell lysates were resolved by SDS/PAGE, transferred to poly(vinylidene difluoride) membranes (NEN), immunoblotted with the indicated antibodies and visualized as described (17). Band intensity was quantified by densitometry by using SCIONIMAGE3B (www.Scioncorp.com).

Luciferase Assay. The 293T cells were transfected with a serum response element (SRE)-luciferase reporter construct and the appropriate Cbl and Lck constructs by using the calcium phosphate method, and assays were performed as described (11).

Results

Lck Ubiquitination and Association on TCR Stimulation. We asked whether coligation of the TCR and CD4 on a normal $CD4^+$ human T cell clone, SPF1, induced Lck ubiquitination. Although Lck ubiquitination was undetectable on CD3 ligation, similar to controls, CD4 ligation resulted in a low level of Lck ubiquitination seen as distinct higher-molecular-weight species together with a smear (Fig. 1*A Upper*). Notably, CD3/CD4 coligation resulted in easily detectable Lck ubiquitination. Anti-Lck immunoblotting revealed the higher-molecular-weight bands and

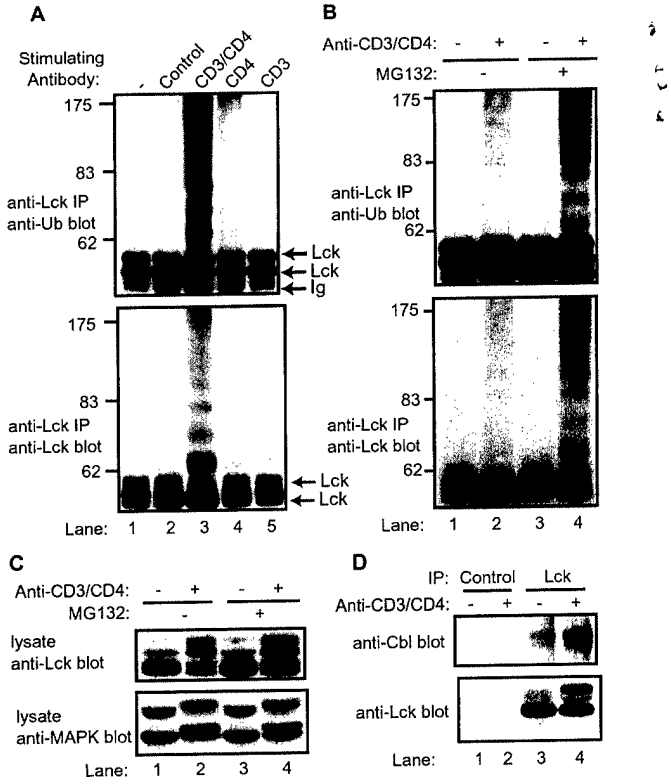

Fig. 1. Lck associates with Cbl and is ubiquitinated upon TCR stimulation in SPF1 T cells. (*A*) Resting $CD4^+$ human SPF1 T cells that had been incubated for 12 h in the absence of IL-2 were stimulated by cross-linking with no antibody (−), control antibody, or anti-CD3/4 antibodies for 10 min at 37°C and then lysed in SDS lysis buffer. Anti-Lck IPs from 1-mg aliquots of lysates was serially probed with an anti-Ub (*Top*) and anti-Lck antibody (*Bottom*). (*B*) SPF1 T cells were treated with 50 μM MG132 (+) or dimethyl sulfoxide vehicle control (−) for 3 h before stimulation for 10 min. IPs were performed and immunoblotted as above. (*C*) Equal amounts of protein lysates from *B* were serially probed with anti-Lck and anti-MAPK antibodies. (*D*) SPF1 T cells were stimulated for 5 min., lysed in Triton lysis buffer, and anti-Lck and isotype matched control IPs were immunoblotted with anti-Cbl (*Top*) or anti-Lck antibody (*Bottom*).

smear to be Lck (Fig. 1*A Lower*, lane 3). The more intense Ub vs. Lck signal on higher-molecular-weight species represents an increased Ub epitope density on multiubiquitinated Lck. Anti-CD3/CD4-induced Lck ubiquitination was also observed by using human lymphoblast preparations freshly derived from peripheral blood (data not shown). The induction of Lck ubiquitination by various stimuli corresponded to their ability to induce early tyrosine phosphorylation events (data not shown).

Pretreatment of SPF1 T cells with the proteasome inhibitor MG132 resulted in a marked enhancement of Lck ubiquitination compared with control (Fig. 1*B Upper*). Concomitantly, MG132 treatment resulted in enhanced detection of the higher-molecular-weight species in an anti-Lck blot (Fig. 1*B Lower*). Anti-Lck immunoblotting of whole cell lysates also revealed that signals corresponding to unmodified Lck species decreased on CD3/4 coligation, apparently reflecting the shift into higher-molecular-weight bands and protein degradation (Fig. 1*C*, lane 2); notably, MG132 treatment led to a slight but reproducible increase in the intensity of this band (Fig. 1*C*, compare lanes 2 and 4). MAPK levels were comparable in the presence and absence of MG132, indicating that changes in Lck protein were specific. Altogether, these findings demonstrate that TCR plus CD4 coligation induces Lck ubiquitination in normal T cells, and that ubiquitination targets Lck to the proteasome.

The Lck SH3 domain binds to Cbl *in vitro*, and the two proteins

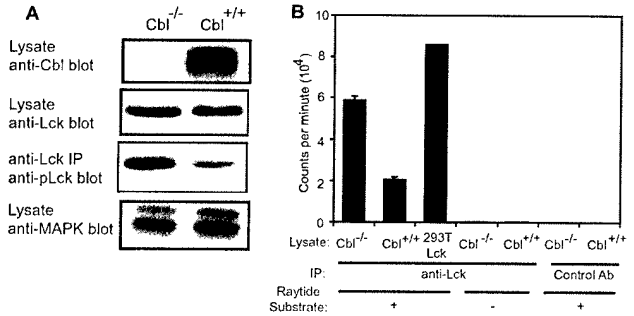

Fig. 2. $Cbl^{-/-}$ T cells have increased levels of kinase-active Lck. (*A*) $Cbl^{-/-}$ and $Cbl^{+/+}$ T cells were lysed in RIPA buffer and equal amounts (50 μg) of protein lysates were immunoblotted with anti-Cbl (top panel), anti-Lck (second panel) and anti-MAPK (bottom panel) antibodies. Anti-Lck IPs from 250 μg of protein lysate was immunoblotted with anti-phospho-Lck (third panel). (*B*) Anti-Lck or isotype matched control IPs from lysates used in *A*, or a positive control lysate from transiently transfected 293T cells, were subjected to *in vitro* kinase assays and the incorporation of ^{32}P signal of [$\gamma^{32}P$]ATP into a synthetic Raytide substrate (+) or negative control substrate (−) was quantified. Results are expressed as the mean ± 1 SD of three replicates.

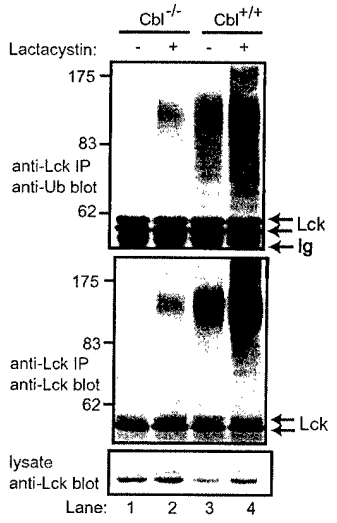

Fig. 3. Impaired Lck ubiquitination in $Cbl^{-/-}$ T cells. $Cbl^{-/-}$ and $Cbl^{+/+}$ T cells were incubated with 10 μM Lactacystin (+) or dimethyl sulfoxide control (−) as well as 0.1 mM orthovanadate for 5 h and then lysed in RIPA buffer. Anti-Lck IPs from 1 mg aliquots of lysate were immunoblotted with anti-Ub antibody (*Top*), followed by anti-Lck antibody (*Middle*). Equal aliquots (30 μg) of cell lysates were immunoblotted with anti-Lck antibody (*Bottom*).

associate *in vivo* (24), suggesting the possibility that Cbl Ub ligase may negatively regulate Lck by means of ubiquitination. In unstimulated SPF1 cells, a low but detectable level of Cbl was coimmunoprecipitated with Lck (Fig. 1*D*, lane 3). Notably, this association substantially increased upon anti-CD3/CD4 stimulation (Fig. 1*D*, compare lanes 3 and 4). Lack of Cbl coimmunoprecipitation in control IPs indicated that the Cbl-Lck association was specific.

Increased Levels of Activated Lck in $Cbl^{-/-}$ T Cells. Given the TCR/CD4-induced Lck ubiquitination and Cbl-Lck association, we asked whether Cbl is required for Lck ubiquitination and degradation by comparing Lck levels in thymocyte-derived, immortalized $Cbl^{-/-}$ and $Cbl^{+/+}$ T cell lines (11). Anti-Cbl immunoblotting of lysates confirmed the expected Cbl deficiency in the $Cbl^{-/-}$ cell line, whereas anti-MAPK immunoblotting showed equivalent sample loading (Fig. 2*A*). Anti-Lck immunoblotting revealed a modestly higher level of total Lck protein in $Cbl^{-/-}$ T cells compared with $Cbl^{+/+}$ cells (Fig. 2*A*, second panel). However, immunoblotting of anti-Lck IPs with an antibody against the phosphorylated activation loop (thus reactive only with activated Lck) revealed a markedly higher level of active Lck in $Cbl^{-/-}$ compared with $Cbl^{+/+}$ cells (Fig. 2*A*, third panel).

To assess directly whether the increased level of autophosphorylated Lck in $Cbl^{-/-}$ cells represented accumulation of kinase-active Lck, anti-Lck IPs were performed with cell lysates (same as Fig. 2*A*) prepared in SDS-containing lysis buffer (to disrupt protein complexes), and subjected to *in vitro* kinase assays. Negligible [^{32}P]ATP incorporation was seen with negative control IPs or if substrate peptide was omitted (Fig. 2*B*). Notably, anti-Lck IPs from $Cbl^{-/-}$ T cell lysates showed 3-fold higher kinase activity than those from $Cbl^{+/+}$ T cells. The accumulation of kinase-active Lck as a result of Cbl deficiency supported a role for Cbl in the ubiquitination and degradation of activated Lck.

Next we asked whether accumulation of Lck in the $Cbl^{-/-}$ T cell line was related to inefficient ubiquitination. A low but detectable Lck Ub signal was observed in $Cbl^{+/+}$ T cells in the absence of the proteasome inhibitor lactacystin; this signal increased markedly on lactacystin treatment (Fig. 3 *Top*, compare lanes 3 and 4). In contrast, the Lck Ub signal was essentially undetectable in $Cbl^{-/-}$ T cells, and the signal remained very low even after lactacystin treatment (Fig. 3 *Top*, compare lanes 1 and 2). The accumulated Lck-Ub could also be visualized with an anti-Lck immunoblot (*Middle*). Furthermore, anti-Lck immunoblotting of whole cell lysate from $Cbl^{+/+}$ cells indicated an accumulation of Lck protein upon lactacystin treatment compared with no change in $Cbl^{-/-}$ cells (*Bottom*). These findings strongly support the conclusion that Lck ubiquitination and protein levels in T cells is controlled by the presence of Cbl protein.

Ubiquitination of Lck in a Reconstitution System. The results in T cells strongly suggested that Lck ubiquitination is a result of its interaction with Cbl. To address this suggestion directly, we compared the ability of the wild-type (WT) Cbl protein vs. the Ub ligase-deficient RING finger mutant C3AHN (23) to target Lck for ubiquitination in transfected 293T cells (Fig. 4*A*). Although relatively little Ub signal was detected on Lck when it was cotransfected with vector (control), coexpression with WT Cbl led to strong Ub signal on Lck, accompanied by a reduction in the level of Lck protein (Fig. 4*A*, compare lanes 1 and 2). In contrast, the Cbl C3AHN mutant was unable to induce Lck ubiquitination or a decrease in Lck protein level (compare lane 2 with lane 3), despite expression at levels comparable with that of WT Cbl (*Bottom*).

Next, we directly tested the role of Lck kinase activity in ubiquitination assays by comparing WT Lck with its kinase active (Y505F) and kinase dead (R273A) mutants (Fig. 4*B*). WT Lck was ubiquitinated and degraded when Cbl was coexpressed, as we had already found. In contrast, constitutively active Lck (Y505F) showed detectable ubiquitination even in the absence of cotransfected Cbl, and this ubiquitination was markedly enhanced when Cbl was coexpressed (compare lane 3 with lane 4). Kinase dead Lck (R273A) was essentially insensitive to Cbl-mediated ubiquitination or degradation (compare lane 6 with lanes 2 and 4). Together, these findings demonstrate that Cbl-mediated ubiquitination of Lck depends on Lck kinase activity and an intact Cbl RING finger domain.

Because Cbl can associate with SFKs through multiple interactions (14), we wanted to determine the relative importance of each interaction for Cbl-mediated degradation of Lck. Coexpression of WT Lck, SH2 mutant (R154K), SH3 mutant

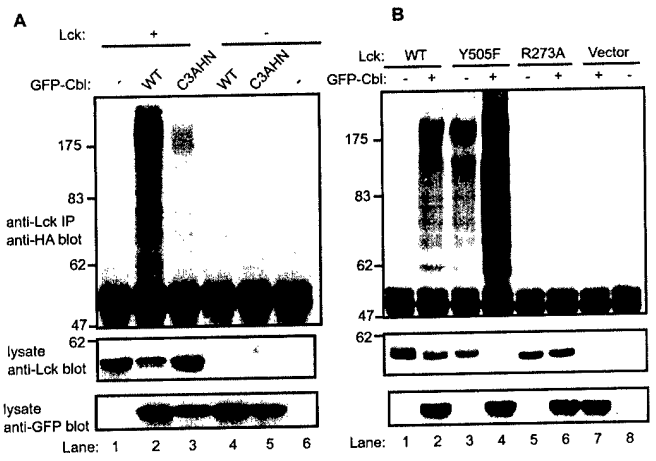

Fig. 4. Cbl-dependent ubiquitination of Lck in 293T cells in dependent on the Cbl RING finger domain and Lck kinase activity. (*A*) 293T cells were transfected with plasmids encoding HA-Ub (5 μg), Lck (0.2 μg), and 3 μg of GFP-Cbl (WT), GFP-Cbl-C3AHN RING finger mutant, or a GFP control (−). Cells were lysed in RIPA buffer, and anti-Lck IPs from 800-μg aliquots of lysate protein were immunoblotted with anti-HA antibody (*Top*). Equal aliquots (30 μg) of cell lysates were immunoblotted with anti-Lck antibody (*Middle*) followed by anti-GFP antibody (*Bottom*). Control GFP is not included in the blot. (*B*) 293T cells were transfected with plasmids encoding HA-Ub (5 μg), Lck (WT), kinase active (Y505F), and kinase dead (R273A) (0.2 μg each), and GFP-Cbl or a GFP control (−) (3 μg). Cells were lysed in RIPA buffer, and immunoblots of anti-Lck IPs were performed as in *A*.

(W97A), or SH3/SH2 double mutant with WT Cbl or the Cbl TKB domain mutant (G306E) followed by coimmunoprecipitation revealed that disruption of the Lck SH3 domain severely abrogated association with Cbl (Fig. 5, compare lane 8 with lane 2). Moreover, disruption of both the SH2 and SH3 domain nearly completely abrogated Lck association with Cbl (compare lane 12 with lane 8). Compared with the SH3 domain mutant, a mutated Lck SH2 domain alone did not significantly disrupt Cbl-Lck association, whereas disruption of the Cbl TKB domain in all

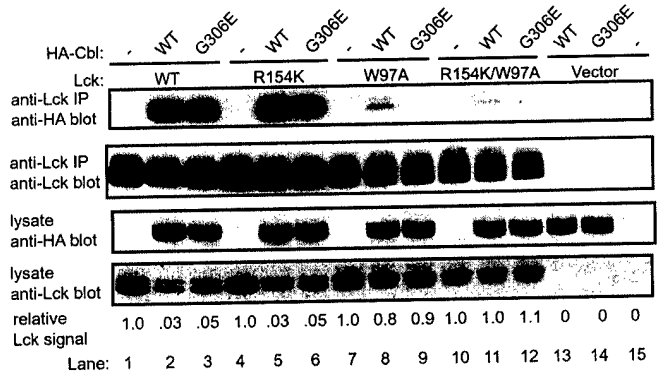

Fig. 5. Relative contribution of Lck SH2 and SH3 domains and the Cbl TKB domain toward Cbl-mediated Lck degradation. 293T cells were transfected with plasmids encoding HA-Cbl (1 μg) and Lck (WT), SH2 (R154K), SH3 (W97A), or double mutants (R154K/W97A) (0.5 μg each). Cells were lysed in Triton lysis buffer and anti-Lck IPs from 1-mg aliquots of lysate protein were immunoblotted with anti-HA antibody (*Top*) and anti-Lck antibody (*Upper Middle*). Aliquots (20 μg) of lysate protein were immunoblotted with anti-HA antibody (*Lower Middle*). For degradation, 293T cells were transfected as above, but with 0.2 μg of the indicated Lck plasmids and lysed with RIPA buffer. 20 μg aliquots of lysate protein were immunoblotted with anti-Lck antibody (*Bottom*). Lck protein levels were quantified by densitometry and are expressed relative to each Lck protein in the absence of coexpressed Cbl.

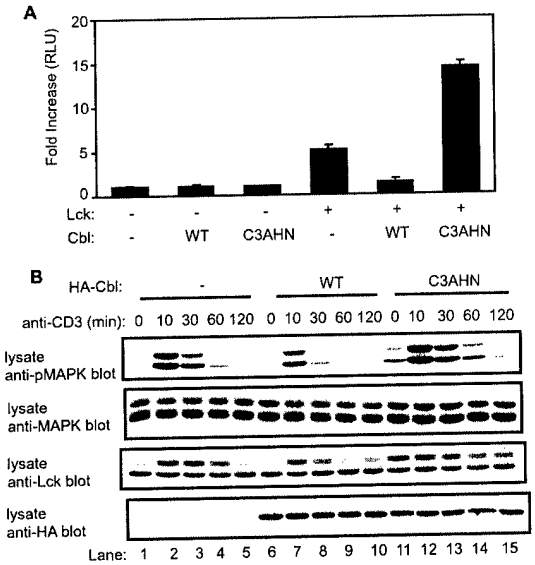

Fig. 6. The RING finger domain is required for Cbl-dependent negative regulation of Lck. (*A*) 293T cells were transfected with plasmids encoding the SRE-luciferase reporter (5 μg) and the indicated combinations of Lck (0.15 μg), HA-Cbl, HA-Cbl-C3AHN and HA-Cbl-70Z (1 μg) or pAlterMAX vector (−). Luciferase activity was expressed relative to activity of lysates transfected with the reporter in the absence of Lck or Cbl. Results represent the mean ± one SD of five replicate transfections. (*B*) Jurkat-derived ZAP-70-deficient p116-T cells, were transfected with 15 μg of plasmid DNA encoding HA-Cbl, HA-Cbl-C3AHN, or pAlterMAX vector (−). Cells were either left unstimulated or stimulated for the indicated times with anti-CD3 antibody before lysis in RIPA buffer. Equal aliquots of cell lysates (25 μg) were subjected to anti-phospho-MAPK (*Top*), anti-MAPK (*Upper Middle*), anti-Lck (*Lower Middle*), and anti-HA (*Bottom*) immunoblotting.

cases slightly decreased Cbl-Lck association. The association data correlated with the ability of Cbl or its TKB domain mutant (G306E) to mediate Lck degradation, as assessed by quantification of Lck protein levels by densitometry (Fig. 5 *Bottom*). Both WT Lck and the SH2 domain mutant were equally sensitive to Cbl-mediated degradation (Fig. 5 *Bottom*, compare lanes 2 and 5). The Lck SH3 mutant was markedly resistant to Cbl-mediated degradation, whereas mutation of both the SH2 and SH3 domains completely blocked degradation (compare lanes 8 and 11). Moreover, the mutation of the Cbl TKB domain slightly blocked degradation of Lck. Together with findings on other SFKs (11, 20), these data suggest that the Lck SH3 domain is the primary mediator of association between Cbl and Lck. The Cbl TKB domain, which specifically binds phosphopeptide motifs (hence phosphorylated Lck), is also involved in this association and subsequent degradation, whereas the Lck SH2 domain plays a much smaller role in Cbl-Lck association and degradation.

The Cbl RING Finger Domain-Dependent Negative Regulation of Lck Function in 293T and Jurkat T Cells. To investigate the functional implications of Cbl-mediated ubiquitination of Lck function, we first compared the effects of WT Cbl and its RING finger domain mutant on Lck kinase-dependent transactivation of the SRE-luciferase reporter (25). Ectopic expression of Lck protein in 293T cells led to a nearly 5-fold increase in SRE-luciferase activity compared with mock-transfected cells (Fig. 6*A*). The Lck-induced increase in SRE-luciferase activity was suppressed to near basal levels upon coexpression of WT Cbl. In contrast, coexpression of the Cbl C3AHN RING finger mutant failed to reduce the Lck-dependent SRE-luciferase reporter activity and, instead, substantially enhanced it (Fig. 6*A*).

Next, we examined the ability of Cbl to regulate Lck function