

# Ubiquitin Ligase Activity and Tyrosine Phosphorylation Underlie Suppression of Growth Factor Signaling by c-Cbl/Sli-1

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## Summary

Receptor desensitization is accomplished by accelerated endocytosis and degradation of ligand-receptor complexes. An in vitro reconstituted system indicates that Cbl adaptor proteins directly control downregulation of the receptor for the epidermal growth factor (EGFR) by recruiting ubiquitin-activating and -conjugating enzymes. We infer a sequential process initiated by autophosphorylation of EGFR at a previously identified lysosome-targeting motif that subsequently recruits Cbl. This is followed by tyrosine phosphorylation of c-Cbl at a site flanking its RING finger, which enables receptor ubiquitination and degradation. Whereas all three members of the Cbl family can enhance ubiquitination, two oncogenic Cbl variants, whose RING fingers are defective and phosphorylation sites are missing, are unable to desensitize EGFR. Our study identifies Cbl proteins as components of the ubiquitin ligation machinery and implies that they similarly suppress many other signaling pathways.

## Introduction

Cellular activation by a large variety of extracellular stimuli is generally followed by transient refractoriness to the same stimulus. One of the better characterized mechanisms of homologous desensitization involves a rapid decrease in the number of the respective cell surface receptors through accelerated endocytosis (Schmid, 1997). This process is termed "downregulation," and it is shared by receptors for growth factors, lymphokines, antigens, and immunoglobulins. The biochemical mechanism underlying receptor downregulation are relatively well understood in the case of tyrosine kinase receptors (RTKs) for growth factors, such as the epidermal growth factor (EGF) and the platelet-derived growth factor (PDGF) (Sorkin and Waters, 1993). Ligand binding to surface receptors activates their efficient internalization via clathrin-coated pits that invaginate to form coated vesicles. Unlike receptors for nutrients (e.g., the transferrin receptor), a significant pool of activated RTKs escapes recycling to the cell surface and is sorted to the lysosome degradation pathway. Sorting of internalized receptors to this pathway is regulated, at least in part, by the intrinsic tyrosine kinase activity of the receptor (French et al., 1994), but it remained poorly understood until very recently. An initial clue has been provided by the invertebrate form of the EGF receptor (EGFR): vulval development in *C. elegans* and development of the R7 photoreceptor in *Drosophila*, two processes that are critically controlled by the EGFR system, are negatively regulated by an adaptor protein called Sli-1/Cbl (Yoon et al., 1995; Meisner et al., 1997).

Sli-1 is an ortholog of the v-cbl transforming gene of the CAS NS-1 retrovirus known to induce pre-B lymphoma and myeloid leukemia (Langdon et al., 1989). Early and prominent tyrosine phosphorylation of the ubiquitously expressed 120 kDa c-Cbl protein was demonstrated in T and B cells stimulated with antigens and in other cell types upon activation by growth factors (e.g., EGF, PDGF, fibroblasts growth factor, colony-stimulating factor, nerve growth factor, prolactin, insulin, and the stem cell factor), cytokines (e.g., interleukin [IL]-2, IL-3, IL-4, interferon- $\alpha$ , erythropoietin, and thrombospondin), fibronectin, and immunoglobulins (for review, see Thien and Langdon, 1998). Overexpression of c-Cbl and transfection of dominant oncogenic mutants uncovered a negative regulatory role of the p120 c-Cbl protein in mammalian cells: not only the EGFR (Levkowitz et al., 1998) and the receptor for PDGF (Miyake et al., 1998) are downregulated upon c-Cbl overexpression, but the function of several immune receptor-coupled tyrosine kinases (e.g., Syk [Ota and Samelson, 1997]) is also suppressed by c-Cbl. Understanding the mechanism by which Cbl proteins downregulate signaling is hampered by the fact that these proteins possess no known catalytic activity. Nevertheless, the functions of several c-Cbl's structural domains have been elucidated. The N-terminal half of Cbl contains a unique Src homology 2 (SH2) domain, which mediates binding to tyrosine-phosphorylated receptors (Meng et al., 1999).

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The C-terminal half carries a long proline-rich domain and several tyrosine phosphorylation sites that mediate constitutive and inducible interactions with kinases (e.g., Src) and guanine nucleotide exchange factors (e.g., Vav, C3G, and SOS). A centrally located C<sub>3</sub>HC<sub>4</sub>-type RING finger (RF) domain separates the two adaptor domains of c-Cbl. A clue to its role may be provided by the fact that the two other family members, Cbl-b and Cbl-3 (Keane et al., 1995, 1999), carry an intact RF. However, this domain is interrupted in the two oncogenic forms of c-Cbl, namely v-Cbl and 70Z-Cbl, an oncoprotein found in a pre-B lymphoma cell line (Andonjou et al., 1994). Consistent with the importance of the RF, mutagenesis of its first cysteine abrogated the suppressive activity (Waterman et al., 1999).

Several recent studies implicate RF domains of other proteins in recruitment of the ubiquitin-proteasome degradation pathways (Tyers and Willems, 1999). The possibility that Cbl proteins may regulate protein ubiquitination through their RF is supported by several studies demonstrating that Cbl overexpression elevates ubiquitination of the receptors for EGF (Levkowitz et al., 1998), PDGF (Miyake et al., 1998), and CSF-1 (Lee et al., 1999). The present study addressed the molecular mechanism underlying Cbl-induced ubiquitination and degradation of RTKs. Assuming a direct role of c-Cbl, we first reconstituted a cell-free system that processes ubiquitination and degradation of isolated EGFRs. Using this reconstituted system, we learned that Cbl is a component of the ubiquitin ligation machinery specific for ligand-activated RTKs. Mutagenesis of the major partners, Cbl and EGFR, unexpectedly revealed the necessity of two tyrosine phosphorylation events for productive ubiquitination and subsequent receptor sorting to degradation: both EGFR and c-Cbl must undergo phosphorylation on specific sites. This requirement represents the first example of the ability of tyrosine phosphorylation to tag proteins for proteasomal degradation.

## Results

### Ubiquitination of an Isolated EGFR and Its Degradation In Vitro Are Directly Regulated by Cbl

c-Cbl can accelerate degradation of EGFR in living cells by increasing receptor ubiquitination (Levkowitz et al., 1998; Waterman et al., 1999). This function is not limited to c-Cbl; the two recently described homologs of c-Cbl, namely Cbl-b (Keane et al., 1995) and Cbl-3 (Keane et al., 1999), are also active. Overexpression of Cbl-b or Cbl-3 in Chinese hamster ovary (CHO) cells led to an accelerated removal from the cell surface of a coexpressed EGFR (Figure 1A and data not shown). Concomitant with accelerated endocytosis, the receptor underwent enhanced ubiquitination and degradation (Figure 1B). Interestingly, an alternatively spliced short variant of Cbl-3 (Cbl-3S), whose SH2 domain is defective, did not affect ubiquitination of EGFR. On the other hand, the two known oncogenic variants of Cbl, v-Cbl and 70Z-Cbl (Langdon et al., 1989), inhibit rather than stimulate the rate of receptor downregulation (Figure 1 and data not shown). Taken together, the results presented in Figure 1 indicate that all three mammalian Cbl proteins are involved in desensitization of EGFR.

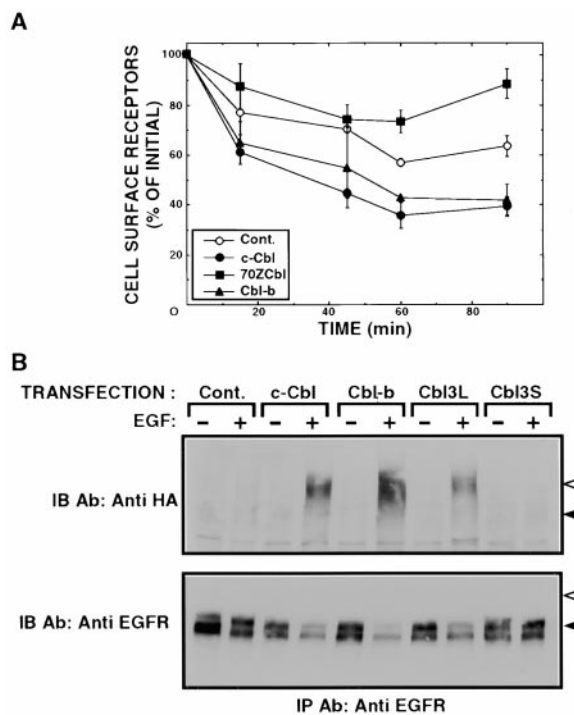


Figure 1. Downregulation and Ubiquitination of EGFR in Living Cells (A) CHO cells were cotransfected with an EGFR expression vector, along with plasmids encoding the indicated Cbl proteins or an empty vector (Cont.). Thereafter, cultures were incubated at 37°C with EGF (100 ng/ml) for the indicated periods of time. Thereafter, cell-bound ligand was removed, and the level of surface receptors was determined in triplicates by binding of a radiolabeled EGF at 4°C. (B) CHO cells were cotransfected with an EGFR expression vector and a plasmid encoding a hemagglutinin (HA)-tagged ubiquitin, along with the indicated c-Cbl, Cbl-b, and Cbl-3 plasmids. Both a short form of Cbl-3 (Cbl-3S) and a long form (Cbl-3L) were tested. Monolayers were incubated for 15 min at 37°C with or without EGF (at 100 ng/ml), and cell lysates subjected to immunoprecipitation (IP) and immunoblotting (IB) with the indicated antibodies. Closed and open arrowheads, respectively, mark the locations of the unmodified and the ubiquitinated forms of EGFR.

To better understand the function of Cbl proteins in receptor ubiquitination and degradation, we aimed at constructing an *in vitro* ubiquitination and degradation system by using recombinant components. A previous attempt to reconstitute EGFR ubiquitination has identified rabbit reticulocyte lysate as a useful source of modifying enzymes (Mori et al., 1997), and our initial utilization of a similar system has attributed an essential role to c-Cbl (Waterman et al., 1999). Two versions of the *in vitro* ubiquitination system are presented in Figure 2A. Both systems use an immunopurified EGFR, a rabbit reticulocyte lysate, ATP, and ubiquitin. Ligation of ubiquitin to EGFR was followed by either labeling of the receptor (with a radioactive phosphate) or by using a radioactive ubiquitin. As we previously reported, bacterially expressed c-Cbl fused to glutathione S-transferase (GST-Cbl) enabled receptor ubiquitination (Figure 2A). Another SH2 protein, namely the Shc protein, was inactive. Control reactions performed in the presence of a large excess of an unlabeled ubiquitin or three other EGFR-interacting proteins, the p85 regulatory subunit of

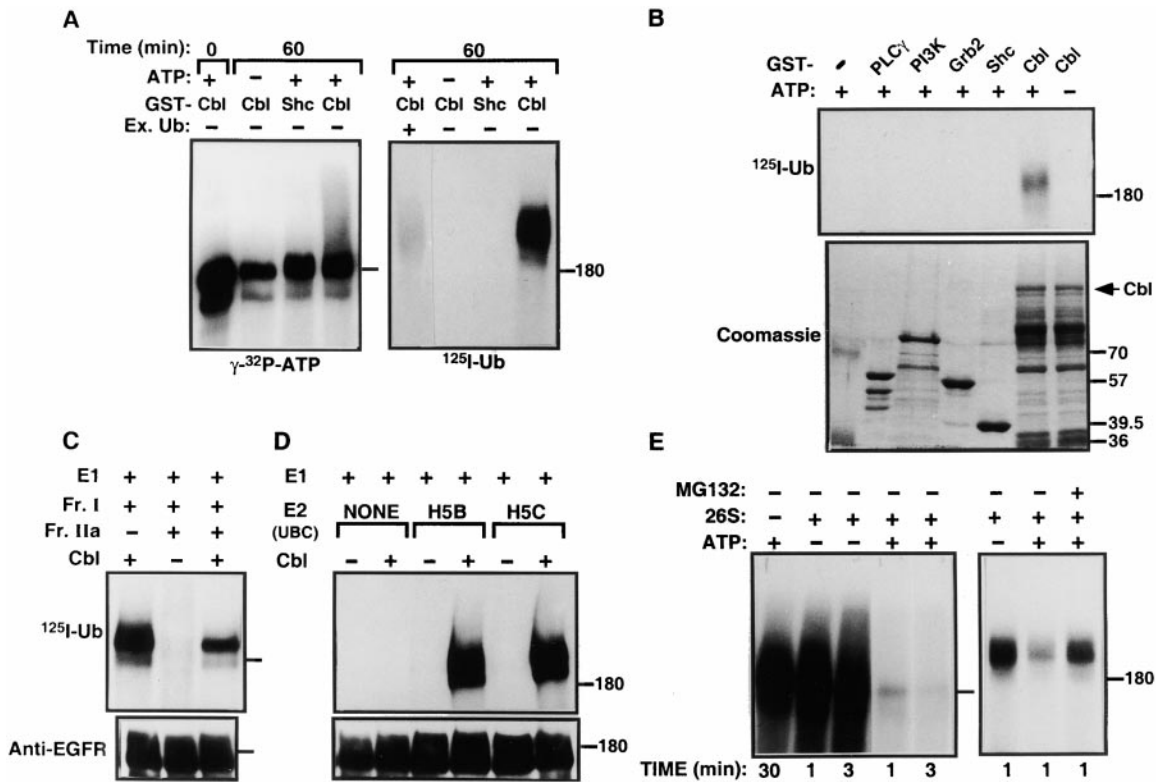


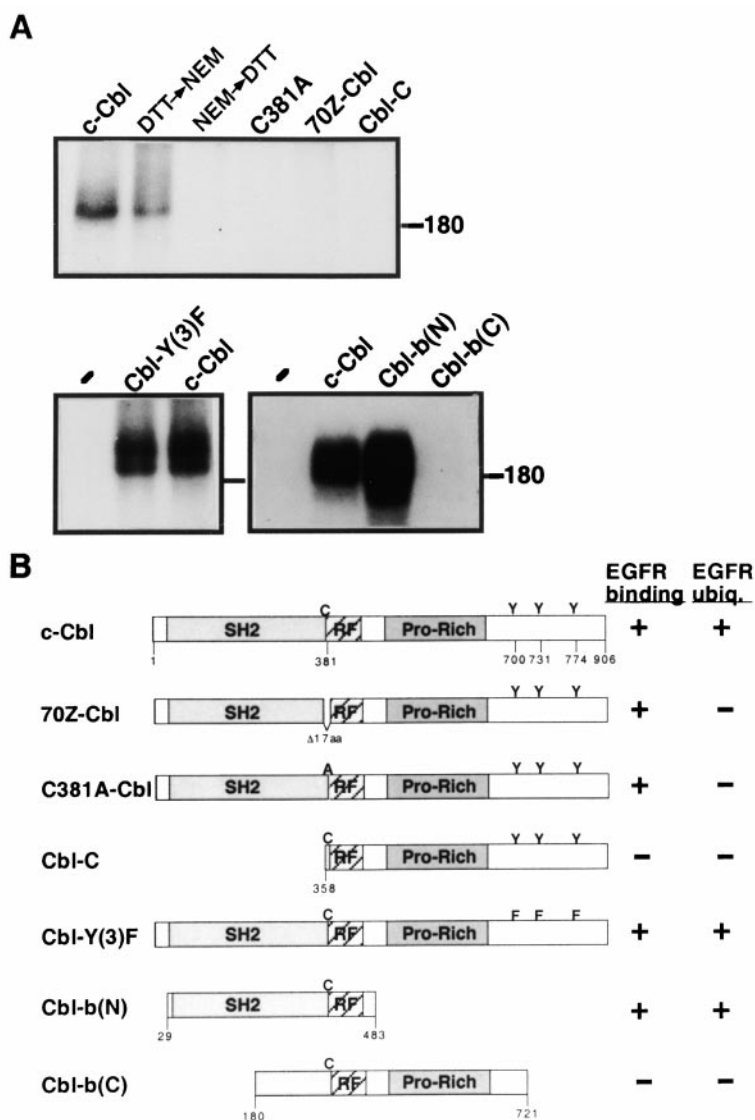
Figure 2. In Vitro Reconstitution of EGFR Ubiquitination and Degradation

(A) (Left) EGFR immunocomplexes were pre-labeled with  $^{32}\text{P}$  and subjected to an in vitro ubiquitination reaction, in the presence of rabbit reticulocyte lysate and the indicated GST fusion proteins, as detailed in the Experimental Procedures. (Right) An in vitro ubiquitination reaction was similarly performed except that an unlabeled EGFR was used and the reaction was performed in the presence of a radiolabeled ubiquitin ( $^{125}\text{I}$ -Ub). Reactions were carried out in the absence or presence of a 100-fold excess of an unlabeled ubiquitin (Ex. Ub). (B) In vitro ubiquitination was performed with a radiolabeled ubiquitin in the presence of one of the following GST fusion proteins: the SH2 domains of PI3K, PLC- $\gamma$ -1, and Shc or full-length Grb-2 and c-Cbl proteins. For control, we used a GST protein alone or omitted ATP, as indicated. The bottom panel shows a stained gel with the respective fusion protein preparations. (C) In vitro ubiquitination was performed as in (B), except that a purified E1 enzyme and the indicated chromatographic fractions of reticulocyte lysates replaced the crude whole lysate. Gel-resolved proteins were transferred to filters, which were first autoradiographed (upper panel,  $^{125}\text{I}$ -Ub) and then immunoblotted with anti-EGFR antibodies. (D) Reactions were performed as in (C) except that recombinant E1 (from insect cells) and the indicated E2 proteins (from bacteria) were used. (E) Purified EGFR was labeled with  $^{125}\text{I}$ -ubiquitin, washed, and incubated at 37°C for the indicated time intervals in the absence or presence of a purified 26S proteasome preparation, ATP, or MG132, as indicated.

phosphoinositide 3-kinase (PI3K), phospholipase C- $\gamma$ -1 (PLC- $\gamma$ ), and Grb-2, confirmed saturability and specificity to Cbl (Figures 2A and 2B).

Although these results indicate that c-Cbl is involved in covalent attachment of ubiquitin to EGFR, they leave open the exact role it plays in the underlying three-step enzymatic reaction. Adenylation of ubiquitin by the ubiquitinous E1 enzyme is followed by transfer of the activated molecule to one of several types of E2 enzymes. These relay ubiquitin either directly or indirectly through an E3 ubiquitin ligase, to the target protein (for review, see Hershko and Ciechanover, 1998). Chromatographic fractionation of whole reticulocyte lysates can separate the major E2 activity (fraction I) from several E3-like activities (fraction IIa) (Abu Hatoum et al., 1998). When tested for ubiquitination of EGFR in the presence of a purified E1, the combination of the two fractions was inactive, but c-Cbl could reconstitute the activity in the presence of E1 and fraction I (Figure 2C). Evidently,

none of the fraction IIa-enriched E3s could replace c-Cbl. Out of five recombinant E2s that we tested, only two enzymes, UBC-H5B and UBC-H5C, were active; UBC-H5A, UBC-H7, and UBC-3/CDC-34 were inactive, as was fraction IIa, which may contain some E2 activity (Figure 2D and data not shown). These results imply that c-Cbl mediates transfer of ubiquitin from a specific E2 to EGFR. Once tagged by ubiquitin, EGFR is destined to intracellular degradation that can be partially inhibited by the proteasomal inhibitor MG132 (Levkowitz et al., 1998). We reconstituted this activity by incubating an in vitro ubiquitinated EGFR with a purified 26S proteasome preparation (Figure 2E). As expected, receptor proteolysis was rapid, ATP dependent, and sensitive to MG132. In conclusion, because c-Cbl can support ubiquitination of EGFR in vitro in the presence of isolated recombinant E1 and E2, the combination of the two modified receptor is processed by the proteasome, c-Cbl probably acts as a ubiquitin ligase or a ligase-ancillary protein.



### The SH2 and RING Finger Domains of c-Cbl Are Necessary and Sufficient for Ubiquitin Ligation to EGFR

The structural determinants of c-Cbl necessary for its ubiquitin-conjugating activity were addressed by testing in vitro a series of GST-Cbl proteins. As expected, the oncogenic 70Z-Cbl protein was inactive in vitro (Figure 3A), although it differed from c-Cbl by only a short stretch of amino acids overlapping part of the RF (see scheme in Figure 3B). On the other hand, an amino-terminal portion of Cbl-b [Cbl-b(N)] retained EGFR binding, and it reconstituted ubiquitination in vitro, indicating that the proline-rich domain and the carboxyl terminus are dispensable for ubiquitin ligation. Lack of direct involvement of the three C-terminal tyrosine phosphorylation sites (Y700, Y731, and Y774 [Feshchenko et al., 1998]) was implied by the ability of a protein mutated at these specific sites, Cbl-Y(3)F, to reconstitute ubiquitination in vitro (Figure 3A). A partially overlapping part of Cbl-b [denoted Cbl-b(C)], as well as an N-terminal

### Figure 3. Structural Requirements of the Cbl-Induced Ubiquitin Conjugation Activity

(A) In vitro ubiquitination reactions were performed with recombinant E1 and E2 (UBC-H5B). To test the effect of alkylation, c-Cbl was treated for 10 min at 22°C with NEM and then with DTT (each at 10 mM) or this sequence was reversed.

(B) The domain structures and in vitro activities of Cbl proteins are shown, and residue numbers are indicated. Boxes correspond to the SH2 domain, a RING finger domain (RF), and a proline-rich domain. Three previously identified tyrosine phosphorylation sites are indicated by Y letters, and a mutant in which all three sites were mutated to phenylalanine (Cbl-3YF) is depicted. An internal deletion of 17 amino acids ( $\Delta 17$  aa) in 70Z-Cbl is also marked. The abilities of Cbl proteins to bind EGFR in vitro and induce its ubiquitination are summarized in a table.

truncated form of c-Cbl (Cbl-C), were inactive, probably because their SH2 domains were defective. These observations implied that a combination of an intact SH2 domain and an uninterrupted RF is sufficient for EGFR modification. We have previously reported that a c-Cbl mutant, whose most N-terminal cysteine of the RF (residue 381) was replaced by an alanine, displays defective functions in living cells (Waterman et al., 1999). We verified lack of activity of the cysteine-to-alanine mutant (C381A-Cbl) in a ubiquitination reaction that uses recombinant E1 and E2 proteins (Figure 3A). This observation is consistent with the notion that the redox potential of many other RING fingers is essential for their activity (Saurin et al., 1996). Indeed, alkylation of c-Cbl with N-ethylmaleimide (NEM) completely abolished its activity, but pretreatment with dithiothreitol (DTT) protected the protein (Figure 3A). A summary of receptor ubiquitination by various Cbl mutants and their ability to bind EGFR in vitro is presented in Figure 3B. As expected, an intact SH2 domain is essential for receptor binding,

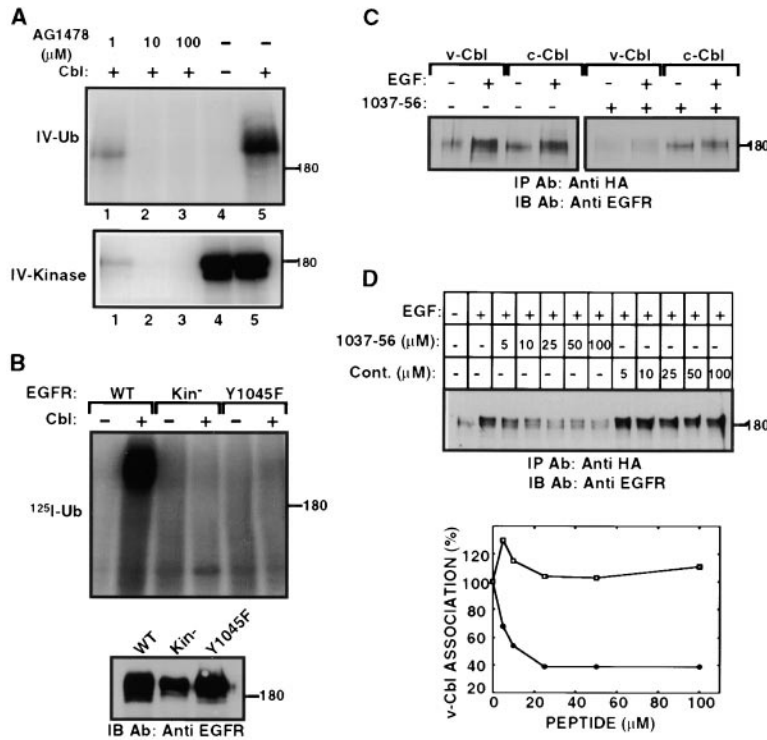


Figure 4. Phosphorylation at Tyrosine 1045 of EGFR Is Necessary for c-Cbl Binding and for Receptor Ubiquitination In Vitro

(A) EGFR immunoprecipitates were washed and pretreated for 20 min at 22°C with the indicated concentrations of AG1478. Thereafter, an in vitro ubiquitination assay was performed in the presence or absence of a recombinant GST-Cbl fusion protein. The bottom panel presents the results of an in vitro kinase assay performed with the corresponding samples in the presence of  $\gamma$ -<sup>32</sup>P-labeled ATP.

(B) Wild type (WT), kinase-defective (Kin<sup>-</sup>), and the Y1045F mutant of EGFR were transiently overexpressed in HEK-293 cells. Receptor immunoprecipitates were subjected to an in vitro ubiquitination assay with a radio-labeled ubiquitin in the presence or absence of GST-Cbl, as indicated. The lower panel shows Western blotting of aliquots of the respective whole-cell lysates.

(C) HEK-293T cells were transfected with vectors directing expression of wild-type EGFR together with plasmids encoding HA-tagged c-Cbl or v-Cbl. Cells were treated for 10 min at 37°C without or with EGF (at 100 ng/ml) 48 hr later. Thereafter, whole-cell lysates were prepared and subjected to immunoprecipitation (IP) with an anti-HA antibody. Where indicated, immunoprecipitation was performed in the presence of a synthetic phosphopeptide

(100  $\mu$ M) corresponding to amino acids 1037–1056 of EGFR. Immunoprecipitates were analyzed by immunoblotting (IB) with anti-EGFR antibodies.

(D) Whole lysates derived from HEK-293T cells transiently expressing EGFR and v-Cbl were mixed with increasing concentrations of the indicated phosphopeptide prior to immunoprecipitation of v-Cbl by using anti-HA antibodies. Immunocomplexes were resolved by gel electrophoresis, transferred to nitrocellulose filters, and the filters blotted with anti-EGFR antibodies. The lower panel shows the densitometry results of the competition assay. Open symbols refer to signals obtained with the control phosphopeptide, and closed circles represent the results obtained with the phosphopeptide flanking tyrosine 1045. Signals were normalized to Cbl binding to EGFR in the absence of peptide.

and it serves as a prerequisite for EGFR ubiquitination. On the other hand, an intact RF is essential for receptor ubiquitination, but the whole C-terminal half of c-Cbl is not necessary for either activity.

#### Phosphorylation at Tyrosine 1045 of EGFR Creates a Major Docking Site for c-Cbl

Although tyrosine phosphorylation of EGFR is essential for stable association of the receptor with c-Cbl, indirect interactions that involve adaptor proteins such as Grb-2 may also link the two proteins (Levkowitz et al., 1996). The availability of a functional in vitro assay of c-Cbl allowed us to test the role of the two types of interactions and also identify the Cbl's docking site. To test the possibility that receptor phosphorylation is necessary for its modification by ubiquitin, we made use of a highly specific inhibitor of the kinase, AG1478 (Gazit et al., 1996). Relatively low concentrations of the antagonist inhibited autophosphorylation of EGFR in a dose-dependent manner (Figure 4A, lower panel), and a parallel decrease in receptor ubiquitination was observed (Figure 4A, upper panel). We then tested the prediction that a kinase-defective EGFR will undergo no ubiquitination in vitro. Indeed, when a wild-type receptor and a kinase-defective mutant were each transiently expressed in HEK-293 human cells and their ubiquitination tested in vitro, we found that only the wild-type form underwent ubiquitination (Figure 4B).

The association between receptor autophosphorylation and ubiquitination in vitro implied that a specific tyrosine autophosphorylation site of EGFR serves as a docking site for c-Cbl. To identify a Cbl-specific docking site, we made use of a series of receptor proteins mutated at individual tyrosines of the carboxyl terminus. Because none of six C-terminal tyrosine residues is necessary for ubiquitination of EGFR in living cells (Levkowitz et al., 1998), we concentrated on the remaining two tyrosine residues (tyrosines 1045 and 1101). The respective mutants were expressed in HEK-293 cells, and the receptors were tested in a ubiquitination assay. This assay revealed that one of the mutants, a receptor whose tyrosine 1045 was changed into a phenylalanine, lost the ability to undergo ubiquitination (Figure 4B). Control experiments verified expression of the mutant receptors and their ability to undergo autophosphorylation (Figure 4B, lower panel, and data not shown). Consistent with the identification of tyrosine 1045, the C-terminally flanking amino acid sequence conforms to the consensus Cbl docking site as characterized with the ZAP-70 tyrosine kinase (Tyrosine-X-X-X-Proline [Meng et al., 1999]). To directly test the docking ability of tyrosine 1045 and the surrounding amino acid sequence, we synthesized the corresponding tyrosine-phosphorylated peptide. The effect of the peptide on the association between EGFR and two Cbl proteins, a wild-type c-Cbl and v-Cbl, was tested in vitro. Because

v-Cbl contains only the SH2 domain, indirect interactions with EGFR, which are mediated by the proline-rich and the C-terminal tyrosines of c-Cbl, are excluded. Figure 4C depicts the results of the association experiment. Evidently, the peptide only slightly affected binding of c-Cbl to EGFR, but it almost completely abolished the interaction with the shorter viral form of Cbl. The specificity of the inhibitory effect was then tested by comparing increasing concentrations of the EGFR phosphopeptide with an unrelated phosphotyrosine peptide of similar length (Figure 4D). Whereas the specific phosphopeptide displaced EGFR from v-Cbl, the control peptide was ineffective. In conclusion, c-Cbl interacts with EGFR in an inducible manner primarily through an autophosphorylated tyrosine 1045 of the receptor.

#### The c-Cbl's Docking Site of EGFR Mediates Ligand-Induced Ubiquitination and Downregulation of the Receptor

Because tyrosine 1045 has not been previously identified as one of the five major sites of EGFR autophosphorylation, we predict it serves as a minor site. Indeed, Western blotting with antibodies to phosphotyrosine detected no significant difference between the content of phosphotyrosine in wild-type and in mutant (Y1045F) forms of EGFR (Figure 5A). However, in line with the results of *in vitro* assays, no basal interaction and only residual ligand-induced association between c-Cbl and the mutant form of EGFR was observed in living cells. Likewise, no up-smearing of EGFR, a characteristic of ubiquitination, was noted with the mutant receptor (Figure 5A). Despite its relatively weak interaction with c-Cbl, the mutant Y1045F receptor retained the ability to increase tyrosine phosphorylation of c-Cbl (Figure 5B). This observation is reminiscent of the ability of a mutant EGFR lacking the whole C-terminal tail to enhance Cbl phosphorylation (Levkowitz et al., 1998). Unlike the interrupted physical association with c-Cbl, mutagenesis of tyrosine 1045 did not interfere with the interaction with another substrate of the EGFR, namely Shc (Figure 5A).

Identification of the Cbl's docking site enabled us to study the role of Cbl-EGFR interactions in living cells. In line with the observation that an overexpressed c-Cbl enhances ubiquitination and degradation of the receptors for EGF (Levkowitz et al., 1998), the Cbl-defective mutant of EGFR (Y1045F) displayed an attenuated downregulation (Figure 5C). Nevertheless, residual ligand-induced downregulation of Y1045F was observed upon Cbl overexpression, indicating the existence of secondary mechanisms of Cbl recruitment to EGFR. The differences between the wild-type and mutant receptor forms were much more prominent when receptor degradation and ubiquitination were analyzed: EGF-induced degradation, as well as ubiquitination, of the mutant receptor in cells overexpressing c-Cbl was almost completely abolished (Figure 5D). Taken together, the results presented in Figure 5 indicate that tyrosine 1045 of EGFR acts as a major c-Cbl docking site, which mediates receptor degradation by enhancing ubiquitination.

#### Tyrosine Phosphorylation of c-Cbl at a Site Flanking the RING Finger Is Essential for an E3-like Ubiquitin Ligase Activity

Although prominent phosphorylation of c-Cbl has been reported in cells stimulated by a variety of ligands (Thien

and Langdon, 1998), the possibility that Cbl phosphorylation regulates its activity has not been addressed before. Our *in vitro* assay of c-Cbl allowed us to examine this scenario. EGFR was isolated, and its autophosphorylation was allowed *in vitro* prior to inhibition of further phosphorylation by using a tyrphostin. Thereafter, c-Cbl was added and receptor ubiquitination tested. Consistent with a requirement for *trans*-phosphorylation of c-Cbl, we observed no receptor ubiquitination under these conditions (Figure 6A). Preincubation of EGFR with c-Cbl prior to adding the inhibitor was sufficient for ubiquitination, probably because *trans*-phosphorylation enabled Cbl activation. The use of two mutants of Cbl provided an initial mapping of the putative Cbl's phosphorylation site: a mutant whose three major C-terminal autophosphorylation sites were defected [Cbl-3(YF)] and a truncated form of Cbl-b, denoted Cbl-b(N), also displayed dependence on prephosphorylation (Figure 6A). Thus, the putative Cbl's site of phosphorylation probably resides in the N-terminal half. To support this conclusion, we prephosphorylated both EGFR and the short form of Cbl-b and then mixed them under conditions that completely block further phosphorylation. As predicted, EGFR ubiquitination could be recovered even when tyrosine phosphorylation was completely inhibited, but prephosphorylation of both EGFR and c-Cbl was absolutely essential (Figure 6B). Incomplete recovery is likely due to the low efficiency of Cbl phosphorylation *in vitro* (Figure 6B, right panel).

To map the site of Cbl, whose phosphorylation is essential for its ubiquitin ligase activity, we concentrated on N-terminal tyrosine residues that are relatively conserved in the Cbl family. Mutagenesis of tyrosines 92, 291, 274, 307, 337, and 368 did not impair the ability of c-Cbl to increase EGFR ubiquitination and degradation in living cells (Figures 6C and 6D). However, a mutant at site 371 completely lost the ability to enhance receptor ubiquitination and degradation in living cells (Figure 6D). Tyrosine 371 flanks the RF of all Cbl proteins, but it is not present in 70Z-Cbl, which explains why this oncogenic variant lacks ubiquitin ligase activity *in vitro* (Figure 3A) and in living cells (Figure 1). In line with the importance of Cbl phosphorylation at this site, a bacterial form of c-Cbl, whose respective tyrosine was mutated, was inactive in an *in vitro* ubiquitination assay (Figure 6E). Control experiments confirmed retention of EGFR binding by the mutant form of c-Cbl. In conclusion, two tyrosine phosphorylation events safeguard productive ubiquitination and subsequent degradation of a ligand-activated EGFR. First, the receptor undergoes tyrosine phosphorylation at a consensus Cbl docking site, and then the ubiquitin ligase activity of the recruited adaptor is activated by phosphorylation at a site flanking the RF.

#### Discussion

Two features are common to all Cbl-associated signaling pathways. First, the function of Cbl is primarily suppressive, and second, all pathways involve tyrosine kinases that physically recruit Cbl and elevate its tyrosine phosphorylation. By developing an *in vitro* ubiquitination assay of EGFR, the present study suggests that Cbl negatively regulates signaling because it recruits active tyrosine kinases to the ubiquitin-proteasome degradative machinery (Figure 2). In addition, our results provide

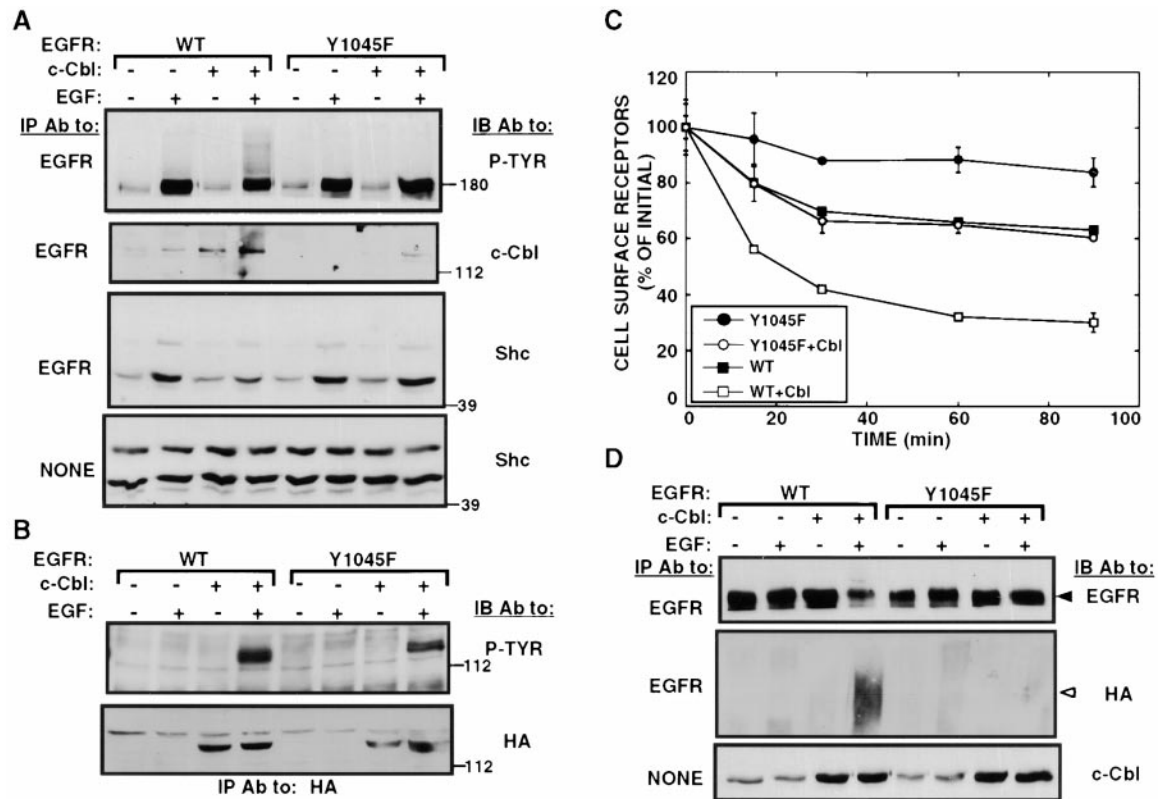


Figure 5. An EGFR Mutated at Tyrosine 1045 Is Impaired in Receptor Downregulation, Ubiquitination, and Degradation

(A) Monolayers of CHO cells were transiently transfected with vectors encoding an HA-tagged c-Cbl or a control empty vector. Cotransfection with plasmids encoding EGFR, either a wild-type receptor or a tyrosine 1045 mutant (Y1045F), was performed as indicated. Following 48 hr of incubation, monolayers were incubated for 10 min at 37°C with or without EGF (100 ng/ml). Whole-cell lysates were subjected to immunoprecipitation with a mAb to EGFR. Subsequent to gel electrophoresis and electrotransfer, filters were immunoblotted (IB) with the indicated antibodies to either phosphotyrosine (P-TYR), Shc, or Cbl. Alternatively, aliquots of the corresponding whole-cell lysates were directly resolved by electrophoresis and then analyzed by using an antibody to Shc.

(B) Cell monolayers were treated as in (A) and the HA-tagged c-Cbl molecules immunoprecipitated by using anti-HA antibodies. Immunocomplexes were resolved by gel electrophoresis and immunoblotting (IB) with the indicated antibodies to phosphotyrosine (P-TYR) or to HA.

(C) Monolayers of CHO cells were transfected with vectors encoding the wild-type form of EGFR or the Y1045F mutant. Along with EGFR plasmids, monolayers were transfected with a c-Cbl-expression vector or a control empty plasmid. Duplicate monolayers were assayed for downregulation of EGFR 48 hr posttransfection.

(D) CHO cells were transiently transfected as in (A) except that a plasmid driving the expression of HA-tagged ubiquitin was included. Following 48 hr, cell monolayers were treated for 10 min at 37°C without or with EGF (100 ng/ml). Receptor degradation and ubiquitination assays were performed as described in the legend to Figure 1B. The lower panel presents immunoblotting of whole-cell extracts with an antibody to c-Cbl. An open arrowhead indicates the location of the ubiquitinated form of EGFR, and a closed arrowhead marks the major unmodified form.

an explanation to the second common feature of Cbl signaling: tyrosine phosphorylation of Cbl is absolutely necessary for its ubiquitin ligase activity toward a substrate tyrosine kinase (Figure 6). Conceivably, the interactions between EGFR and c-Cbl may be summarized in a sequential model (Figure 7). Steps 1 and 2 involve two distinct tyrosine phosphorylation events. Initially, an autophosphorylation reaction creates docking sites for several signaling proteins, including a Cbl binding site at tyrosine 1045 of EGFR. Second, EGFR *trans*-phosphorylates Cbl at a linker domain, which activates an associated ubiquitin ligase activity. Interestingly, only the N-terminal half of c-Cbl (see Figure 3B) is necessary for recruitment of an active E2 and for the ensuing substrate ubiquitination (step 3). This conclusion is consistent with the shorter structures of the two known invertebrate forms of Cbl, as well as with the structure of Cbl-3 (Keane et al., 1999). Below, we discuss two major unresolved aspects of the proposed model. First,

we refer to analogous phosphorylation-dependent ubiquitin ligation systems and deal with the mechanism enabling c-Cbl to mediate substrate ubiquitination. Second, we address the possibility that the various steps of EGFR-Cbl interactions take place along the journey of EGFR to the lysosome.

#### Ubiquitin Ligase Activity of Cbl Proteins

To achieve target specificity, the ubiquitin-proteasome system selects its substrates in a highly regulated manner (Hershko and Ciechanover, 1998). Of the three-step ubiquitin transfer cascade, the E3-mediated ligation step is the most variable and specific. To safeguard selectivity, multiprotein E3 complexes, whose association often involves phosphorylation events, are assembled at the substrate. The potential role of Cbl proteins in such E3 complexes that target activated tyrosine kinases to degradation may be illuminated by the analogy to one of the best characterized modular E3 complexes

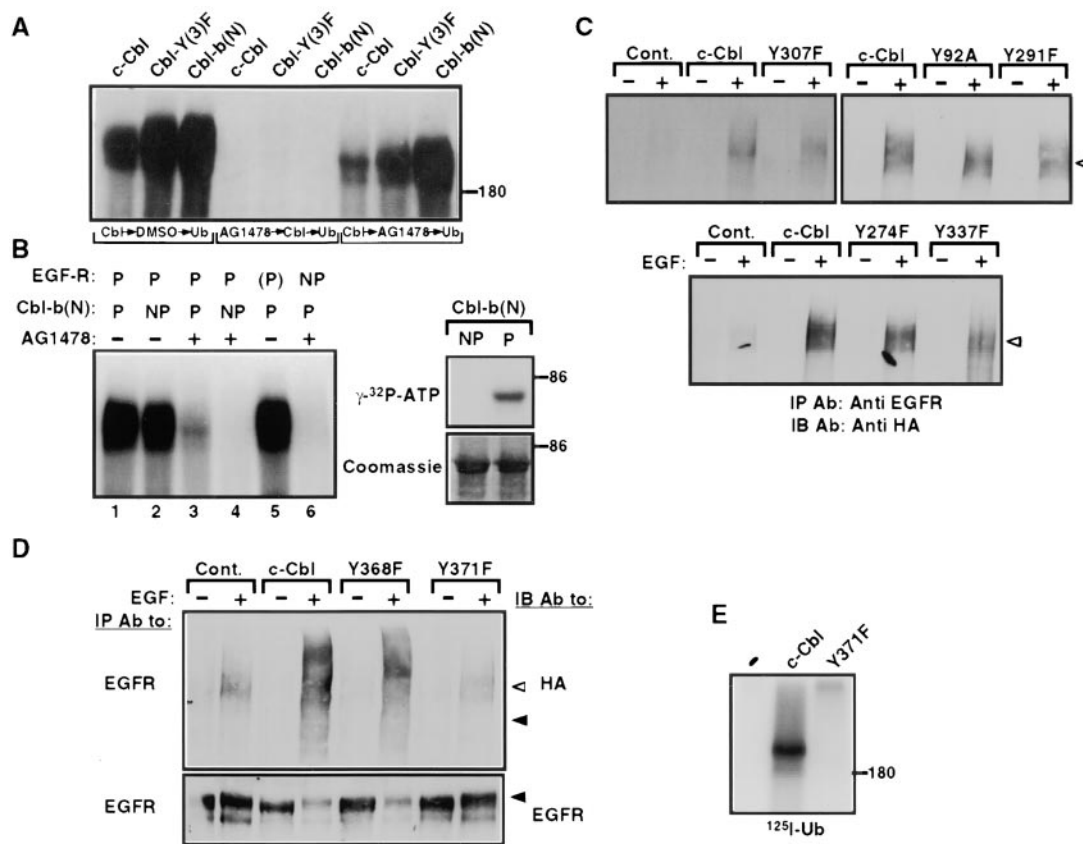


Figure 6. Tyrosine Phosphorylation of Cbl Proteins Is Essential for EGFR Ubiquitination In Vitro and in Living Cells

(A) An in vitro ubiquitination reaction of EGFR was performed with the indicated GST-Cbl proteins. An immunopurified EGFR was first preincubated for 20 min at 4°C with ATP under conditions that allow receptor autophosphorylation, and then we added either AG1478 (50 μM, or solvent dimethylsulfoxide [DMSO] for control) or a GST-Cbl protein. The order of adding the kinase inhibitor and the Cbl proteins is indicated at the bottom.

(B) GST-Cbl-b(N) was immobilized on glutathione-agarose and subjected to phosphorylation (marked by P letters) by preincubation with a membrane preparation from A-431 cells. Likewise, EGFR was used either in its phosphorylated (P) or nonphosphorylated (NP) form and mixed with aliquots of Cbl-b(N). AG1478 (50 μM) was added as indicated prior to an in vitro ubiquitination reaction. In the lane labeled by (P), EGFR was not subjected to prephosphorylation, but kinase activity was not inhibited (lane 5). To verify in vitro phosphorylation, Cbl-b(N) was similarly treated in the presence of radioactive ATP (right panel). Dye staining of the corresponding lanes is also shown.

(C) CHO cells were cotransfected with an EGFR expression vector and a plasmid encoding a hemagglutinin (HA)-tagged ubiquitin, along with plasmids encoding the indicated Cbl proteins. As control, we used an empty expression vector (Cont.). Each monolayer was split into two separate plates 24 hr after transfection, and 24 hr later identical sister plates were incubated for 15 min at 37°C without or with EGF (100 ng/ml). Cell lysates were subjected to immunoprecipitation (IP) with an anti-EGFR antibody, followed by immunoblotting (IB) with an antibody directed to HA.

(D) A ubiquitination assay of EGFR in living cells was performed as described in (C), except for the use of plasmids encoding c-Cbl proteins mutated at tyrosines 368 or 371. The lower panel shows the results of immunoblotting with an anti-EGFR antibody

(E) An in vitro ubiquitination assay of EGFR was performed in the presence of the indicated mutant Cbl proteins.

of the SCF (Skp1-Cdc53/Cul1-F box) type (Maniatis, 1999). Both IκB, an inhibitory subunit of the NF-κB transcription factor, and the transcriptional coactivator β-catenin are recognized by a receptor called β-TRCP (Yaron et al., 1998). This receptor binds the substrates through its WD40 domain, which recognizes vicinal phosphoserine residues. Another domain, the F box, binds a second component of the SCF, Skp1, which recruits a third subunit, Cdc53/CUL1 (reviewed in Maniatis, 1999). Similar modular complexes mediate degradation of a variety of other proteins, including mitotic cyclins and hypoxia-induced proteins (reviewed in Tyers and Willems, 1999), all sharing a recently identified fourth subunit, Rbx1/Apc11/Hrt1, which encompasses an RF (Seol et al., 1999, and references therein). Like β-TRCP and other receptor subunits of E3 complexes,

Cbl proteins physically associate with their ubiquitination substrates in a phosphorylation-dependent manner. Therefore, we raise the possibility that Cbl is functionally equivalent to a combination of β-TRCP and Rbx1. Our finding that the segment linking the SH2 and RF domains of Cbl must be modified suggests a conformational change that activates the E3 complex, in analogy to the Rbx1 component of the VHL-type E3 complex (Kamura et al., 1999).

While it is presently unknown whether or not Cbl recruits E3 ancillary proteins in a phosphorylation-dependent manner, it seems likely that the RF mediates recruitment of the E2 enzyme. This assumption is based upon the occurrence of RF domains in many complexes that mediate substrate ubiquitination (Tyers and Willems, 1999), and the recently observed direct binding of an

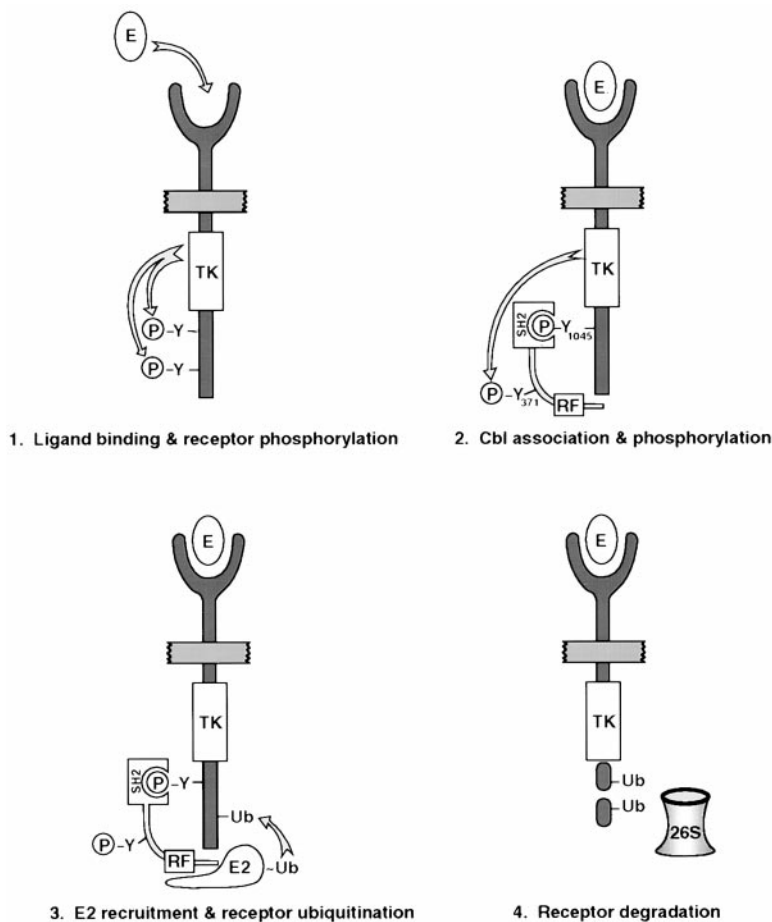


Figure 7. Proposed Sequential Process Leading to EGFR Degradation

Binding of EGF (E) to the extracellular part of EGFR stimulates the tyrosine kinase (TK) domain and results in elevated phosphorylation of several C-terminally located tyrosines, including tyrosine-1045 (step 1). The latter serves as a docking site for the SH2 domain of c-Cbl. Once a stable EGFR-Cbl complex is formed (step 2), the adaptor protein undergoes phosphorylation at a region linking the SH2 and the RING finger (RF) domains. This phosphorylation event allows, through an unknown mechanism, recruitment of a ubiquitin-loaded E2 molecule (step 3). Then, E2 relays its thioester-bound ubiquitin to EGFR, thereby enabling recruitment of the 26S proteasome, and proteolysis of the cytoplasmic portion of EGFR (step 4). Apparently, the four steps occur while the receptor translocates from the plasma membrane, via an endosomal compartment, to a prelysosomal structure, where the luminal ectodomain is exposed to lysosomal hydrolases.

E2, called UBCM4, to a set of RF proteins (Martinez-Noel et al., 1999). Despite the unknown functions of Cbl phosphorylation, it is clear that the Cbl-associated ubiquitin ligase activity is essential for desensitization of signaling processes. One exemplification is provided by oncogenic strategies that apparently corrupt the normal process of EGFR degradation: the essential phosphorylation site of Cbl at tyrosine 371 is deleted in the oncogenic 70Z-Cbl form, and the viral *cbl* gene encodes only an SH2 domain without an RF. On the other hand, the site of EGFR that mediates Cbl binding is frequently deleted in *erbB* oncogenes of avian erythroblastosis virus strains (Lee et al., 1993). Deletion of this site may inhibit inactivation of the virally encoded form of EGFR and thereby prolong cellular activation. Consistent with the transforming effect of blocking EGFR-Cbl interaction, the respective artificial mutants of either Cbl (Andoniou et al., 1994) or EGFR (Lee et al., 1993; Traverse et al., 1994) are characterized by enhanced signaling.

#### Relationships between Endocytosis and Cbl-Induced Ubiquitination of Activated Receptors

Ubiquitin conjugation to substrate proteins almost invariably targets them to degradation by the proteasome (Hershko and Ciechanover, 1998). Accordingly, proteasome inhibitors block intracellular degradation of several growth factor receptors, whose ligands induce elevated ubiquitination. Included in this list are EGFR (Levkowitz et al., 1998) and the receptor for PDGF (Mori

et al., 1995). An alternative role for receptor ubiquitination originally emerged from studies of the yeast  $\alpha$  factor receptor (Hicke and Riezman, 1996). Ubiquitination of this receptor plays a causative role in its endocytosis. The situation in mammalian cells is less clear: preventing internalization of the growth hormone receptor abolished its ubiquitination (Govers et al., 1997), and Cbl<sup>-/-</sup> macrophages, which are defective in ubiquitination of the colony stimulating factor-1 receptor, retain endocytosis of the ligand (Lee et al., 1999). Identification of tyrosine 1045 of EGFR as the c-Cbl docking site (Figure 4) enabled us to approach the relationships between ubiquitination and receptor endocytosis.

An EGFR mutant whose interaction with c-Cbl was defected exhibited no ubiquitination and degradation, and it apparently remained at the cell surface following stimulation with EGF (Figure 5). However, closer analyses of receptor's fate and ligand degradation revealed that the mutated receptor underwent internalization, that was followed by release of the ligand and recycling back to the cell surface (H. W. et al., unpublished data). Preliminary morphological analyses suggest that recycling occurs already from the early endosome. Because degradation of EGFR is mediated primarily by lysosomal hydrolases (reviewed in Sorokin and Waters, 1993), it seems likely that ubiquitination of EGFR plays a critical role in sorting of internalized receptor molecules to the lysosome. By using other EGFR mutants (Levkowitz et al., 1998) and an RF-defective form of c-Cbl (Waterman

et al., 1999), we have previously localized the sorting effect of c-Cbl to the transition from early to late endosomes. This conclusion is in agreement with several other observations. First, progressive deletions from the carboxyl terminus of EGFR identified residues 1022–1123, a segment that includes tyrosine 1045, as a lysosomal targeting motif (Kornilova et al., 1996). When deleted, the truncation mutant exhibited enhanced recycling and a minimal extent of sorting to the late endosome. Second, a kinase-defective mutant of EGFR, which is devoid of ubiquitination (Figure 4B), slowly internalizes but recycles back to the cell surface upon reaching the early endosome (Felder et al., 1990). It is interesting to note that the Cbl-docking site we identified is found in two ErbB proteins (ErbB-1 and ErbB-2) that undergo ubiquitination and lysosomal degradation (Galcheva Gargova et al., 1995; Mimnaugh et al., 1996). However, it is absent in ErbB-3, a receptor that undergoes no ubiquitination and is constitutively recycled from early endosomes to the plasma membrane (Waterman et al., 1998). Taken together, these lines of evidence support the possibility that ubiquitination of EGFR targets incoming receptors to the late endosome, a compartment where both proteasomal and lysosomal hydrolases may respectively degrade the cytoplasmic and exoplasmic domains.

In summary, the present report identifies a novel activity of Cbl proteins that enables them to assemble the ubiquitination machinery at an activated tyrosine kinase, while sparing the nonstimulated form of growth factor receptors. Previous works have demonstrated that serine phosphorylation of the substrate (e.g., I $\kappa$ B and  $\beta$ -catenin [Aberle et al., 1997]) or the E3 complex itself (e.g., the cyclosome [Lahav-Baratz et al., 1995]) can strictly regulate ubiquitin ligation and subsequent degradation. Cbl-mediated degradation of EGFR presents a unique example in which tyrosine phosphorylation of both the substrate (EGFR) and the E3 complex (Cbl) regulates ubiquitin ligation. Future studies will address the existence of putative additional components of the Cbl-containing E3 complex and the role it plays in targeting endocytosed EGFRs to specific vesicular compartments.

#### Experimental Procedures

##### Materials and Antibodies

Radioactive materials were purchased from Amersham (Buckinghamshire, United Kingdom). Iodogen was from Pierce. MG123 was from Calbiochem. AG1478 was a gift from A. Levitzki (Hebrew University, Jerusalem, Israel). An antibody to the Shc protein was purchased from Transduction Laboratories. Anti-hemagglutinin (HA) monoclonal antibody 12CA5 and yeast hexokinase were purchased from Boehringer-Mannheim (Mannheim, Germany). Murine monoclonal antibody (mAb) SG565 to EGF receptor was generated in mice that were immunized with a recombinant extracellular portion of human EGFR. For immunoblot analysis of EGFR, we used a polyclonal antiserum from Santa Cruz Biotechnology (Santa Cruz, CA). Synthetic phosphotyrosine peptides with the following sequences were prepared by using standard procedures: KEDSFLQRpYSSDP TGALTED (EGFR) and IDIFSDpYANFKAKKK (protein tyrosine phosphatase epsilon, a gift from A. Elson).

##### cDNA Constructs and Expression of Recombinant Fusion Proteins

Mammalian expression plasmids for EGFR, c-Cbl, 70Z-Cbl, Cbl-b, and Cbl-3 (long and short forms) were previously described (Levkowitz et al., 1998; Ettenberg et al., 1999; Keane et al., 1999). The

Y1045F mutant of EGFR was prepared by mutagenizing a single strand template. Bacterial GST-Cbl and a GST-70Z-Cbl expression vector were generated by replacement of Cbl's first ATG codon with a BstPEI site and insertion of a BstPEI-SalI fragment into the compatible XmaI and XhoI sites of pGEX-4T2 (Pharmacia). Other GST-Cbl proteins were made in bacteria by constructing similar pGEX vectors. The RF mutant of Cbl (C381A) was described (Waterman et al., 1999). GST-Cbl fusion proteins were affinity purified as we previously described (Levkowitz et al., 1996). Cloning and expression of UBC-H5B and UBC-H5C were described elsewhere (Jensen et al., 1995). Recombinant E1 was produced in Baculovirus-infected Sf-9 cells, purified on an ubiquitin-agarose column, and eluted with adenosine monophosphate.

##### Receptor Downregulation and Ubiquitination Assays

Receptor downregulation assays were performed as described (Levkowitz et al., 1998). The ubiquitinated form of EGFR was detected in immunoprecipitates prepared from cells that were cotransfected with a plasmid encoding an HA-tagged ubiquitin (a gift from Dirk Bohmann, EMBL, Heidelberg, Germany) and an EGFR expression vector. The receptor was immunoprecipitated from whole-cell lysates, and its ubiquitination levels were determined by immunoblotting with anti-HA antibodies.

##### In Vitro Assays of Cbl Binding, Receptor Ubiquitination, and Phosphorylation

EGFR was immunoprecipitated from cleared lysates of A-431 cells by using an agarose-immobilized mAb SG565 as described (Waterman et al., 1999). Following purification, agarose beads were extensively washed and resuspended in buffer containing 40 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 2 mM DTT, 2 mM ATP- $\gamma$ -S, and 3  $\mu$ g/ml <sup>125</sup>I-labeled ubiquitin (or 0.4 mg/ml unlabeled ubiquitin). To deplete endogenous ATP, hexokinase (1 mg/ml) and 2-deoxyglucose (20 mM) were also included. Crude rabbit reticulocyte lysate (5  $\mu$ l, from Promega) or the previously described (Abu Hatoum et al., 1998) fraction I (250  $\mu$ g), fraction IIA (50  $\mu$ g), purified E1 (2  $\mu$ g), or recombinant E1 (1  $\mu$ g) and E2 (7  $\mu$ l of crude bacterial extract) were added as indicated. Reaction mixtures were supplemented with a GST-Cbl protein (5  $\mu$ g) and incubated for 1 hr at 30°C. The beads were then extensively washed and EGFR eluted with gel sample buffer. In vitro phosphorylation of an immunoprecipitated EGFR was performed by using a 20 min long incubation on ice with buffer containing 20 mM HEPES-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 10 mM MnCl<sub>2</sub>, and 5  $\mu$ Ci  $\gamma$ -<sup>32</sup>P-ATP. Immunoprecipitates were then washed and resolved by gel electrophoresis. To test binding of Cbl proteins to EGFR, we prepared whole lysates from EGF-stimulated A-431 cells and incubated them for 1 hr at 4°C with immobilized GST-Cbl proteins. Thereafter, the associated EGFR was detected by gel electrophoresis and immunoblotting.

##### In Vitro Degradation Assay of EGFR

Cell-free degradation was performed following EGFR conjugation with a radiolabeled ubiquitin in a reaction mixture containing a purified 26S proteasome preparation (1  $\mu$ g) (Ben-Shahar et al., 1999) in buffer containing 40 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 2 mM DTT, 4 mM ATP, 2 mg/ml ovalbumin, 20  $\mu$ g/ml ubiquitin-aldehyde and a broad-specificity protease inhibitor cocktail (Calbiochem). ATP depletion and inhibition of proteasomal activity were respectively attained by preincubation of the proteasome preparation for 10 min at 37°C with either hexokinase (1 mg/ml) and 2-deoxyglucose (20 mM) or with MG132 (12.5  $\mu$ M).

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