The RING Finger of c-Cbl Mediates Desensitization of the Epidermal Growth Factor Receptor*

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Ligand-induced activation of surface receptors, including the epidermal growth factor receptor (EGFR), is followed by a desensitization process involving endocytosis and receptor degradation. c-Cbl, a tyrosine phosphorylation substrate shared by several signaling pathways, accelerates desensitization by recruiting EGFR and increasing receptor polyubiquitination. Here we demonstrate that the RING type zinc finger of c-Cbl is essential for ubiquitination and subsequent desensitization of EGFR. Mutagenesis of a single cysteine residue impaired the ability of c-Cbl to enhance both down-regulation and ubiquitination of EGFR in living cells, although the mutant retained binding to the activated receptor. Consequently, the mutant form of c-Cbl acquired a dominant inhibitory function and lost the ability to inhibit signaling downstream to EGFR. In vitro reconstitution of EGFR ubiquitination implies that the RING finger plays an essential direct role in ubiquitin ligation. Our results attribute to the RING finger of c-Cbl a causative role in endocytic sorting of EGFR and desensitization of signal transduction.

We have reported recently that c-Cbl can accelerate the rate of EGFR degradation by increasing conjugation of polyubiquitin to an endocytosed EGFR (1). Consistent with sorting to degradation, genetic studies in Caenorhabditis elegans attributed a negative regulatory role to Sli-1, the c-Cbl ortholog of worms (2). This function is reminiscent of the action of c-Cbl downstream to the PGDFR (3), and it depends on a catalytically intact kinase domain of EGFR. The present study addressed the structural motif of c-Cbl involved in ubiquitination of EGFR. Although the 120-kDa c-Cbl protein possesses no identifiable catalytic activity, its NH2-terminal half carries a novel phosphotyrosine-binding domain (PYB) (4), that presumably mediates binding to the activated receptor (5). The carboxy-terminus of c-Cbl comprises several proline-rich tracts that allow constitutive binding of Src homology 3 (SH3)-containing proteins, such as Grb2, Nck, and the Cbl-associated protein, CAP (reviewed in Ref. 6). In addition, the COOH terminus is involved in inducible interactions with SH2-containing proteins, such as the regulatory subunit of the phosphoinositide 3-kinase, and the guanine nucleotide exchange protein Vav. An evolutionarily conserved RING-type zinc finger domain separates the adaptor domains of c-Cbl. RING fingers (RFs) are zinc-binding domains that differ from other zinc finger motifs in terms of structure and the zinc ligation scheme (reviewed in Ref. 7). Noteworthy is the fact that the two other family members of c-Cbl carry an intact RF (8, 9), but oncogenic Cbl variants are defective in the RF (10, 11). Our present study concentrated on the role the RF motif plays in Cbl-mediated degradation of EGFR.

EXPERIMENTAL PROCEDURES

Materials, Buffers, and Antibodies—EGF was purchased from Sigma, and radioactive materials were from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). Rabbit anti-c-Cbl (C-15) antibodies, anti-EGFR, and a monoclonal antibody (mAb) to phosphotyrosine were from Santa Cruz Biotechnology (Santa Cruz, CA). A mAb to the active doubly phosphorylated form of Erk was from Sigma. The compositions of TBST, HNTG, binding-binding and solubilization buffers were as described (12). Ubiquitin was radiolabeled by using IODOGEN (Pierce) and Na125I.

Construction and Transfection of Expression Vectors—We have previously described the construction of c-Cbl, 70Z-Cbl, and v-Cbl in the pCDNA3 expression vector (Invitrogen) containing the HA sequence tag (13). To generate an aminoterminal fragment of c-Cbl, we used the Stratagene Quick-change mutagenesis kit. The ubiquitin-hemagglutinin A (Ub-HA) expression vector was a gift from Dirk Bohmann (EBI, Heidelberg, Germany). The protocols for transfection of CHO cells were exactly as described (1). A bacterial pGEX4T2 expression vector (Amersham Pharmacia Biotech) was used for expression of GST-Cbl proteins. GST-fusion proteins were affinity-purified on glutathione agarose as described previously (13).

Lysate Preparation, Immunoprecipitation, and Western Blotting—Whole cell lysates were cleared by centrifugation (12,000 × g, 10 min) and analyzed either directly or after immunoprecipitation by SDS-PAGE and immunoblotting as described (12). Protein bands were detected with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Receptor Down-regulation and Phosphorylation Assays—Down-regulation of EGFR was assayed as described (12). For in vitro kinase assays, EGFR immunoprecipitates were incubated for 15 min on ice in HNTG containing 5 mM MnCl2 and 0.01 μCi of [γ-32P]ATP. The immobilized immunocomplexes were then washed and resolved by SDS-PAGE.

SRE Transcription Assay—Transfection of the indicated vectors was performed in triplicates with a reporter pSRE-Fluc plasmid (4 μg), containing one copy of the SRE cloned upstream to the Fos minimal promoter (nucleotides −53/+45) and the luciferase gene (14). Twenty-four hours later, cells were treated with EGF (20 ng/ml) or left untreated for 12 h. Whole cell extracts were prepared by resuspending the cells in 30 μl of 1 × lysis buffer (Promega). Cellular debris were removed by a 10-μl aliquot mixed with 100 μl of luciferin buffer (0.1 μ Tris acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, 74 mM luciferin, and 2.2 μM ATP). Light intensity was measured by using a luminometer. The results were normalized to protein concentrations.

In Vitro Ubiquitination Assay—EGFR was immunoprecipitated from

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‡ The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; CHO, Chinese hamster ovary; GST, glutathione S-transferase, Ub, ubiquitin; HA, hemagglutinin; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; RF, RING finger; SH, Src homology; SRE, serum response element; ATP-5′-O-(thiotriphosphate); DCC, deleted in colon cancer; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.

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A-431 cell lysates with an agarose-immobilized mAb SG565 and used as a substrate. Following purification, agarose beads were extensively washed and resuspended in a buffer containing 40 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2 mM dithiothreitol, 2 mM ATP, and 100 μM-labeled ubiquitin (5 μM/mL). To deplete endogenous ATP, we added hexokinase (1 mg/mL) and 2-deoxyglucose (30 mM). Crude rabbit reticulocyte lysate (5 μL, from Promega) was added to the reactions. Reaction mixtures were supplemented with GST fusion proteins as indicated (5 μg) and incubated for 1 h at 30 °C. The beads were then extensively washed and EGFR eluted with SDS-PAGE sample buffer prior to electrophoresis.

RESULTS AND DISCUSSION

Mutagenesis of the RING Finger Impairs Cbl-induced Down-regulation of EGFR—The recently reported ability of c-Cbl to enhance degradation of the EGFR (1) and the platelet-derived growth factor receptor (3) can explain the phenotype of Sli-1 mutant worms (2), but they leave open the mechanism underlying increased ubiquitination and degradation. For several reasons we suspected that the RF domain of c-Cbl is involved in ubiquitination. First, the centrally located RF is flanked by non-functional domains, but its own activity is unknown. Second, the RF-defective oncogenic forms of c-Cbl, namely: the CAS NS-1 retrovirus-encoded v-Cbl (11) and the 70Z-Cbl of pre-B cell lymphoma (10), cannot mediate accelerated degradation of EGFR (1). Third, the RFs of several proteins, for example the corresponding region of the DCC (deleted in colon cancer)-binding protein, Siah (15), can promote proteolysis via the ubiquitin-proteasome pathway. To test involvement of the RF, we mutated cysteine 381, the first cysteine of the C₅HC₄ zinc finger motif, to an alanine. This mutation is expected to completely disrupt site-1 of the cross-brace zinc ligation motif (7). The ability of the mutant protein, which we denoted C381A-Cbl, to affect ligand-induced removal of EGFR from the cell surface (“down-regulation”) was compared with that of the wild type c-Cbl and its two oncogenic variants. CHO cells expressing no endogenous EGFR were co-transfected with an EGFR expression vector, along with Cbl-encoding plasmids. The results presented in Fig. 1A demonstrate that while c-Cbl overexpression caused remarkable acceleration of ligand-induced clearance of EGFR from the cell surface, the two oncogenic forms of c-Cbl as well as the RF mutant could not enhance receptor disappearance (Fig. 1A). To substantiate the effect on down-regulation, we examined the effect of C381A-Cbl on EGFR degradation and ubiquitination. In accord with our previous results, c-Cbl overexpression induced both limited degradation of EGFR and up-smearing of the protein band (Fig. 1B, upper panel). Both C381A-Cbl and 70Z-Cbl were inactive. Western blotting with antibodies to epitope-tagged ubiquitin molecules confirmed that the up-smearing effect of c-Cbl was due to extensive ubiquitin ligation to EGFR (Fig. 1B, middle panel). As expected, both C381A-Cbl and 70Z-Cbl were unable to induce receptor ubiquitination. Moreover, the basal ubiquitination of EGFR was slightly decreased in the presence of 70Z-Cbl. Despite its inability to induce down-regulation, degradation, and ubiquitination, C381A-Cbl retained physical association with EGFR following ligand stimulation, as was shown by a co-immunoprecipitation experiment (Fig. 1B, lower panel). Likewise, both mutant Cbl proteins exhibited enhanced tyrosine phosphorylation in response to ligand stimulation. In addition, their basal phosphorylation was significantly higher than that of the wild type c-Cbl (Fig. 1C, middle panel). The results obtained with the point-mutated Cbl protein indicate that the RF is critically involved in ligand-induced degradation of EGFR and in the preceding ubiquitination step.

C381A-Cbl Inhibits c-Cbl-induced Receptor Ubiquitination—The ability of the RF mutant of c-Cbl to form a complex with EGFR (Fig. 1B) and to undergo ligand-induced tyrosine phosphorylation (Fig. 1C) implied that it may inhibit interaction of the wild type form of c-Cbl with ligand-stimulated EGFR. Ligand-induced ubiquitin ligation to EGFR was selected as a functional read-out of the interactions between c-Cbl and the activated receptor (1). CHO cells transiently co-overexpressing c-Cbl and EGFR exhibited ligand- and c-Cbl-dependent ubiquitination of EGFR (Fig. 2, upper panel). This effect was significantly inhibited upon co-transfection with a plasmid encoding the indicated Cbl proteins. An empty vector was used for control (Cont.). All transfections were carried out in the presence of an expression vector encoding a tagged ubiquitin molecule (Ub-HA). Cell monolayers were treated for 10 min at 37 °C without or with EGFR (100 ng/ml). Thereafter, cell lysates were prepared and analyzed by immunoprecipitation (IP) and immunoblotting (IB) with the indicated antibodies. C. CHO cells were transfected as described above except that the Ub-HA plasmid was omitted and anti-HA antibodies were used to detect c-Cbl. The open arrow indicates the position of c-Cbl.

![Fig. 1. Effect of C381A-Cbl on down-regulation, ubiquitination, and degradation of EGFR.](image)
Consistent with the effect on the SRE, overexpression of c-Cbl almost abolished basal and ligand-induced MAPK activity. Both C381A-Cbl and 70Z-Cbl were unable to block MAPK activation (Fig. 3B). In fact, the two proteins weakly, but reproducibly, enhanced the effect of EGF, in accord with their inhibitory effect on down-regulation. It is worth noting that a previous work (16) reported on the ability of 70Z-Cbl to enhance recruitment of several components of the Ras-MAPK pathway (e.g. Grb2, Shc, and Sos1), but no effect on Erk activity was detectable.

**Recombinant c-Cbl Can Mediate in Vitro Ubiquitination of the EGFR, but the RING Finger Mutant Is Inactive**—The involvement of the RF of c-Cbl in ubiquitination of EGFR in living cells (Figs. 1 and 2) and the recent influx of reports on the direct involvement of RFs in degradation of various substrates (15, 20, 21) prompted us to test C381A-Cbl in a cell-free system. To reconstitute in vitro a c-Cbl-dependent ubiquitin ligation reaction, we immunoprecipitated EGFR from human epidermoid A-431 carcinoma cells, where the receptor is highly overexpressed. The antibody-receptor complexes were extensively washed and then supplemented with bacterially expressed c-Cbl, 70Z-Cbl, or C381A-Cbl fused to GST. To follow its covalent ligation to EGFR we radiolabeled ubiquitin and supplemented the reaction mixture with a reticulocyte lysate, a well charac-

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**Fig. 2.** The C381A-Cbl mutant inhibits EGFR ubiquitination induced by the wild type form of c-Cbl. CHO cells were transiently co-transfected with an EGFR expression vector (2 μg) along with an Ub-HA plasmid (1 μg) and increasing concentrations of a c-Cbl vector. Transfections were performed in the presence of plasmids encoding C381A-Cbl or 70Z-Cbl, as indicated (5 μg each). For control we used an empty vector. Forty-eight hours later cell monolayers were treated for 10 min at 37 °C with or without EGF (100 ng/ml). Subsequently, cell lysates were analyzed by immunoprecipitation with anti-EGFR antibodies and immunoblotting with anti-HA antibodies. The 180-kilodalton region of the gel is shown.

**Fig. 3.** The RING finger of c-Cbl is involved in desensitization of EGFR signaling. A, CHO cells were co-transfected in triplicates with pcDNA3-EGFR (1 μg) along with plasmids encoding the indicated Cbl proteins (each at 1 μg). For control we used empty vectors. All transfections were carried out in the presence of the SRE-luc reporter plasmid (4 μg). Thirty-six hours later cells were untreated or treated with EGF (20 ng/ml). Following twelve additional hours the cells were harvested for a luciferase assay. Signals obtained were normalized to protein concentrations and are presented as average ± S.D. B, CHO cells were transiently transfected with an EGFR expression vector along with plasmids encoding the indicated Cbl proteins or a control plasmid. Cell monolayers were stimulated with EGF (100 ng/ml) for the indicated time intervals at 37 °C. Whole cell lysates were analyzed with an antibody specific to the active doubly phosphorylated form of MAPK.
were active ubiquitin by means of adenylation. As expected from our stud-
ties, the absence of ATP, probably due to the necessity to activate oth-
er proteins, either constitutively (e.g. Grb2) or in an inducible manner (e.g. Crk and 14-3-3) may regulate the RF-mediated ubiquitin ligation reaction. Potentially, Cbl regulation may also involve redistribution within the cytoplasm because a fraction of the protein translocates into EGFR-loaded endo-
somes upon activation by EGF (1). c-Cbl regulation may now be addressed by using the dominant negative mutant we de-
scribed in this report. Moreover, the in vitro ubiquitination assay we established appears useful for studying c-Cbl and its mutants and homologues in a cell-free system (23). Future studies will have to address the molecular mechanisms under-
lying RF-induced recruitment of the ubiquitination machinery to
tyrosine-phosphorylated substrates and the role played by the
many Cbl-associated proteins.

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**FIG. 4.** The RING finger domain of Cbl mediates ubiquitina-
tion of isolated EGFR molecules in vitro. **Upper panel,** immuno-
purified EGFR was incubated for 60 min at 30 °C with whole rabbit reticu-
loeyte lysate, 125I-ubiquitin, and the indicated GST-Cbl fusion
proteins. For control we used bacterial GST alone. An ATP analog was
added as noted. At the end of incubation, the immobilized EGFR was
washed and resolved by electrophoresis. The resulting autoradiogram is
shown along with the location of a 180-kilodalton marker protein (my-
osin). **Lower panel,** immunocomplexes were subjected to an in vitro
kinase assay, using [γ-32P]ATP as indicated. Reactions were performed
in the presence of the indicated GST-fusion proteins or GST alone. The
resulting autoradiogram is shown.

Cbl-mediated Desensitization of EGF Receptor

Regardless of the exact role of C-Cbl in the cascade of ubiqui-
quitin transfer reactions, our study clearly indicates that an
intact RING finger domain is essential for this modification of
EGFR. Taken together with previous reports, our present stud-
iess propose a structure-function picture of c-Cbl and its family
members, Cbl-b (8) and Cbl-3 (9), which share with c-Cbl an
extremely well conserved RF. Thus, the NH2- and COOH-
terminal halves of Cbl act as adaptor domains that bind a
phosphotyrosine and SH2/3 proteins, respectively, whereas the
intervening RF mediates ubiquitination of specific substrates.