Ubiquitin Ligase Activity of c-Cbl Guides the Epidermal Growth Factor Receptor into Clathrin-coated Pits by Two Distinct Modes of Eps15 Recruitment*

We have demonstrated previously that c-Cbl requires the presence of a functional ubiquitin interacting motif (UIM) in Eps15 to mediate epidermal growth factor receptor (EGFR) internalization. Both the ubiquitin ligase activity of c-Cbl and the UIM of Eps15 were necessary for plasma membrane recruitment of Eps15 and entry of ligand-bound EGFR into coated pits and vesicles containing Eps15. This is consistent with a scenario in which ubiquitin moieties appended to activated EGFR complexes act as docking sites for Eps15 and thereby recruit receptors into clathrin coated pits. Here, we have investigated which additional structural features of c-Cbl are required for this process. We find that c-Cbl can guide ligand-bound EGFR into the Eps15 internalization route by two distinct mechanisms. These are either dependent on the phosphotyrosine binding domain of c-Cbl that directly binds to the EGFR or on the region C-terminal of the Ring finger, which allows for indirect binding to an alternative site on the receptor. No strict requirement exists for either ubiquitin modified EGFR or the Cbl binding ubiquitination substrate CIN85 as docking site for the UIM of Eps15. Only in the phosho-tyrosine binding-dependent pathway, the EGFR is ubiquitinated and may serve as a site of recruitment for Eps15. Only in this pathway, Eps15 is tyrosine-phosphorylated, but this appears unrelated to its capacity to participate in EGFR internalization.

Attenuation of growth signals occurs by removal of pertinent receptors from the cell surface by endocytosis and their degradation in lysosomes. Either constitutively or upon ligand binding, receptors cluster in plasma membrane specializations such as clathrin-coated pits (CCP), which subsequently invaginate and pinch off to form endosomes (1). The importance of growth factor receptor down-regulation is highlighted by the malignant cell transformation that results from constitutive overexpression of the EGFR or its relatives (2).

It was first found in yeast that mono-ubiquitin can serve as an internalization and/or sorting signal for plasma membrane receptors (3, 4). In mammalian cells, membrane receptors can be ubiquitinated as well. In particular, tyrosine kinase-linked receptors are ubiquitinated by a family of proteins called Cbl (5–8). The members of this family, c-Cbl, Cbl-b, and Cbl-c (9–12), all contain an N-terminal phosphotyrosine binding (PTB) domain that allows direct interaction with activated receptor tyrosine kinases (13). Their second conserved feature is a Ring finger that binds ubiquitin conjugating enzymes and thereby classifies the Cbl family as ubiquitin ligase (14–17).

We and others (18, 19) have previously demonstrated that the EGFR is ubiquitinated at the plasma membrane prior to its internalization via clathrin-coated pits and vesicles. Recent studies indicate that this concerns mono-ubiquitination at multiple sites, rather than poly-ubiquitination. Moreover, use of an EGFR-ubiquitin chimera showed that a single ubiquitin moiety suffices to support receptor endocytosis and degradation (20, 21). This pointed at the existence of a ubiquitin-driven internalization pathway for the EGFR. A number of data suggested a role for Eps15 in such a pathway. First, Eps15 has a functional UIM (22, 23). Second, Eps15 is recruited to the plasma membrane upon EGFR stimulation (24) and can localize to coated pits (25). Finally, Eps15 is involved in clathrin coat assembly, possibly by targeting AP-2 to the plasma membrane (26–30). Therefore, we examined whether the ubiquitin ligase function of c-Cbl served to recruit Eps15 to the activated EGFR and to promote thereby its redistribution into CCP.

Our findings appeared consistent with a model based on studies in yeast (31), in which the ubiquitinated EGFR is recruited to CCP by interaction with the UIM of Eps15. We showed that the ubiquitin ligase function of c-Cbl was required for EGFR-induced recruitment of Eps15 to the plasma membrane, where it co-localized with ligand-bound EGFR (32). Importantly, mutation of the UIM of Eps15 abrogated the capacity of c-Cbl to promote Eps15 membrane recruitment and EGFR internalization into Eps15-containing endosomes. In the absence of the ubiquitin-UIM connection, the EGFR could still be internalized in a clathrin-dependent manner, but this proceeded via an endosomal pathway that was devoid of Eps15 (32). Our findings support a scenario in which Eps15 is recruited to the plasma membrane upon EGFR stimulation because its UIM binds to a protein within the EGFR complex that is ubiquitinated by c-Cbl. This would subsequently guide the activated EGFR into a clathrin-dependent endocytic route involving Eps15 (Fig. 1A). However, direct ubiquitin-dependent interaction of Eps15 with the EGFR could not be demonstrated. Therefore, the Eps15 docking protein might be either the EGFR or an alternative molecule.

In the present study, we have examined which structural properties of c-Cbl, apart from its Ring finger, are required for internalization of the EGFR via the ubiquitin-driven Eps15

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‡ The abbreviations used are: CCP, clathrin-coated pits; EGF, epidermal growth factor; EGFR, EGF receptor; BSA, bovine serum albumin; CHO, Chinese hamster ovary; IP, immunoprecipitate; mAb, monoclonal antibody; pAb, polyclonal antibody; PTB, phosphotyrosine binding; TCI, total cell lysate; TxR, Texas Red; UIM, ubiquitin interacting motif; HA, hemagglutinin; PBS, phosphate-buffered saline.

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route. In particular, we have addressed the role of the N-terminal PTB domain, which allows for direct binding to phospho-tyrosine Tyr(P)\textsuperscript{1045} in the activated EGFR (15) and the role of the C-terminal region that is present in c-Cbl and Cbl-b but not Cbl-c. The C-terminal region harbors tyrosine phosphorylation sites and proline-rich sequences and can interact with a number of signaling proteins, among which are the adaptors Grb-2 and CIN85 (33–35) (Fig. 1B). Via Grb-2, Cbl proteins can bind to the EGFR-independent of their PTB, at another site than Tyr(P)\textsuperscript{1045} (36). CIN85 is known to affect EGFR internalization, which may be related to its capacity to recruit endophilin, a protein involved in membrane curvature (37). Alternatively or in addition, the fact that CIN85 is mono-ubiquitinated by c-Cbl (38) may imply a role in recruitment of UIM-containing endocytic proteins such as Eps15. Our findings indicate that c-Cbl can mediate EGFR internalization via the Eps15 route by two distinct mechanisms, which are either dependent on its PTB or its C terminus.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Antibodies**—COS-7 and Chinese hamster ovary (CHO) cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with fetal calf serum, glutamine and antibiotics. The following antibodies were used: rabbit polyclonal serum 282.7 directed against the EGFR (for blotting); Dr. L. H. DeFize, Hubeckratory Laboratory, Utrecht, The Netherlands; the mouse monoclonal antibody (mAb) 528, directed against the EGFR (for precipitation; American Type Culture Collection); mouse mAb 12CA5 and HA-7 (Sigma) against the HA tag (for blotting); rat mAb 3F10 against the HA tag (for florescence; Roche Applied Science); mAb M2 directed against the FLAG-tag (Sigma); rabbit polyclonal antibody (pAb) H-896 directed against Eps15 (Santa Cruz Biotechnology); rabbit pAb C-15, directed against c-Cbl (Santa Cruz Biotechnology); mAb 4G10 against Tyr(P) (Upstate Biotechnology, Lake Placid, NY). For enhanced immunofluorescence, horseradish peroxidase-conjugated anti-rabbit and anti-mouse or swine anti-rabbit Ig (Dako, Glostrup, Denmark) were used. For immunofluorescence, affinity purified fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and Cy5-conjugated goat anti-rat IgG (Rockland, Gilbertsville, PA) were used.

**Constructs**—The pMT2 expression vectors containing cDNA encoding the human EGFR, HA-tagged ubiquitin, HA-tagged c-Cbl (human), or HA-tagged c-CblR829A or G306E point mutation have been described previously (18, 32). The R829A or G306E point mutation was introduced in the pMT2HA-c-Cbl vector using the QuikChange site-directed mutagenesis kit (Stratagene). Using this method, the G306E mutation was also introduced in the pMT2HA-HA-c-Cbl vector. The HA-tagged c-CblΔC cDNA, encoding amino acids 1–481, was obtained by digestion of pMT2-HA-c-Cbl with Eagle and cloned into the Bgl II site of pMT2HA. The HA-coding sequence was introduced after digestion with XhoI by filling in the overhangs with the Klenow fragment of DNA polymerase. The v-Cbl construct, encoding amino acids 1–357, was made by PCR, using pGEM 4Z-c-Cbl (a gift from W. Y. Langdon, University of Western Australia) as a template and cloned into pMT2HA. The FLAG-tagged wild-type mouse Eps15 cDNA was a gift from E. Klapisz and F. van Bergen en Henegouwen (University of Utrecht, Utrecht, The Netherlands) and was cloned into pMT2. Correct cloning and mutagenesis of all mutants was verified by sequencing.

**Transfections**—COS cells (1 × 10⁶ per 60-cm² dish) were transfected using FuGENE (Roche Applied Science) according to the manufacturer’s instructions. Per well, a total of 2 μg of DNA was used in a ratio of 1:6 to the FuGENE solution.

**Immunoprecipitation and Immunoblotting**—Cells were used for immunoprecipitation 48 h after transfection. COS cells were serum-starved overnight, CHO cells for 3 h, and stimulated with EGF for 2 min at 37 °C. To remove antibodies from the blot, it was incubated for 30 min at 50 °C in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 100 mM NaCl, 1 μM EDTA, 0.1% Triton X-100, 10 mM SDS, 1% sodium deoxycholate, 1% NaN₃, 25 mg/ml sodium thiosulfate, 0.1% aprotinin, and 1% leupeptin). Proteins were visualized using enhanced chemiluminescence (Amer sham Biosciences, Roosendaal, The Netherlands). To remove antibodies, the blot was washed for 30 min at 50 °C in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 100 mM NaCl, 1% NaN₃, 25 mg/ml sodium thiosulfate, 0.1% aprotinin). The following antibodies were used: rabbit polyclonal serum 282.7 directed against the EGFR, HA-tagged ubiquitin, HA-tagged c-Cbl (human), or HA-tagged c-CblR829A or G306E point mutation have been described previously (18, 32). The G306E mutation was also introduced in the pMT2HA-c-Cbl vector using the QuikChange site-directed mutagenesis kit (Stratagene). Using this method, the G306E mutation was also introduced in the pMT2HA-HA-c-Cbl vector. The HA-tagged c-CblΔC cDNA, encoding amino acids 1–481, was obtained by digestion of pMT2-HA-c-Cbl with Eagle and cloned into the Bgl II site of pMT2HA. The HA-coding sequence was introduced after digestion with XhoI by filling in the overhangs with the Klenow fragment of DNA polymerase. The v-Cbl construct, encoding amino acids 1–357, was made by PCR, using pGEM 4Z-c-Cbl (a gift from W. Y. Langdon, University of Western Australia) as a template and cloned into pMT2HA. The FLAG-tagged wild-type mouse Eps15 cDNA was a gift from E. Klapisz and F. van Bergen en Henegouwen (University of Utrecht, Utrecht, The Netherlands) and was cloned into pMT2. Correct cloning and mutagenesis of all mutants was verified by sequencing.

**RESULTS**

**Structure and Function of Cbl Domains**—We have established previously that the capacity of c-Cbl to promote EGFR internalization via the Eps15 route depended on its ubiquitin ligase activity, which is determined by its Ring finger. The well characterized mutant 70Z-Cbl selectively lacks the Ring finger domain, while v-Cbl lacks the Ring finger and in addition all C-terminal sequences (Fig. 1B). To address the role of other c-Cbl domains in internalization of the EGFR via the Eps15 route, we made use of a number of mutants, which lacked certain N- or C-terminal features (Fig. 1B). Wild-type Cbl molecules share an N-terminal PTB domain, which is composed of a four-helix bundle, an EF-hand and an Src homology 2-like domain (15). With this PTB, Cbl molecules bind to receptor tyrosine kinase systems, such as the EGFR, upon their tyrosine phosphorylation following receptor triggering (Fig. 1A). Phosphotyrosine binding by this domain is disrupted by mutation of the glutamate residue at position 306 (c- CblG306E) (39–41). 70Z-CblG306E in addition lacks the Ring finger. In contrast, CblΔC has an intact PTB and Ring finger, but it lacks the C-terminal part and therefore does not bind proteins that normally associate with proline-rich sequences or phosphorylated tyrosine motifs in this region. Among these is the adaptor protein Grb-2, which binds to proline-rich regions in the C terminus of Cbl and in this way can link Cbl to the EGFR via an alternative Tyr(P) docking site (36). The c-CblR829A mutant selectively lacks the binding site for the CIN85 adaptor protein, which is a substrate for mono-ubiquitination by c-Cbl and plays a role in c-Cbl-mediated receptor internalization (35, 37).

**c-Cbl Only Ubiquitinates the EGFR When It Binds via Its PTB, Not When It Binds via Its C-terminal Region**—First, we assessed the role of c-Cbl domains in EGFR ubiquitination. COS cells, which endogenously express EGFR, were co-trans-
fected with HA-tagged ubiquitin and empty vector (control) or vector encoding different HA-tagged Cbl molecules. Immunoblotting on total cell lysates (TCLs) showed that similar amounts of HA-tagged Cbl were expressed in the different transfected cell populations (Fig. 2, lower panel, HA)). Cells were stimulated with EGF for 0 or 15 min, and the EGFR was immunoprecipitated from the cell lysates. Activation of the EGFR was confirmed by anti-Tyr(P) blotting on these IPs and anti-EGFR blotting confirmed that similar amounts of EGFR were isolated from the different cell populations (Fig. 2, lower panel).

Anti-HA blotting on EGFR IPs revealed both HA-tagged ubiquitin and transfected Cbl molecules. Clearly, EGFR stimulation resulted in EGFR ubiquitination in control cells, which had not been transfected with Cbl (Fig. 2, upper panel). This is due to the activity of endogenous c-Cbl, which is recruited to the EGFR complex (Fig. 2, lower panel, cCbl). Transfection and consequent overexpression of wild-type c-Cbl resulted in enhanced recruitment of c-Cbl molecules to the EGFR and thereby in enhanced EGFR ubiquitination as compared with the control situation (Fig. 2, upper panel). In contrast, overexpression of the c-CblG306E mutant with a disrupted PTB domain resulted in enhanced recruitment to EGFR but did not increment EGFR ubiquitination like wild-type c-Cbl (Fig. 2, upper panel). The C-terminal sequences of c-Cbl appeared dispensable for EGFR ubiquitination, since the CblΔC mutant enhanced EGFR ubiquitination over control levels to the same extent as wild-type c-Cbl (Fig. 2, upper panel). In the case of CblΔC its recruitment to the EGFR is difficult to see. In the anti-HA blot, the band representing this mutant migrated close to the Ig heavy chain of the antibody used for immunoprecipitation. Anti-Cbl antibody cannot recognize this mutant and therefore only reveals endogenous c-Cbl. We conclude that transfected c-Cbl lacking a functional PTB cannot promote EGFR ubiquitination. Lack of the C-terminal region, however, does not affect the capacity of Cbl to ubiquitinate the EGFR.

We also tested Ring finger mutants of c-Cbl for their capacity to exert a dominant negative effect on EGFR ubiquitination by endogenous c-Cbl. The 70Z-Cbl mutant, which has both an intact PTB and an intact C-terminal region, completely over-
ruled the function of endogenous c-Cbl and reduced EGFR ubiquitination to undetectable levels (Fig. 2, upper panel). Interestingly, the v-Cbl mutant, which can only bind to the EGFR via the PTB and not via Grb-2, since it lacks the C-terminal region, was dominant negative to the same extent as 70Z-Cbl. In contrast, 70Z-CblG306E, which has a disrupted Ring finger, but lacks the PTB, could not inhibit EGFR ubiquitination by endogenous c-Cbl (Fig. 2, upper panel). As compared with transfected wild-type c-Cbl, the Ring finger mutants were more abundantly present in EGFR complexes, which suggests reduced turnover of Cbl in the absence of its ubiquitin ligase function. We conclude that endogenous c-Cbl cannot induce EGFR ubiquitination when v-Cbl blocks the PTB binding site on the receptor. In contrast, blocking of the alternative binding site on the EGFR by the 70Z-CblG306E mutant has no such effect. Collectively, these data indicate that, under these experimental conditions, the EGFR is only ubiquitinated when c-Cbl binds to the activated receptor via its PTB but not when it binds via its C-terminal region.

Tyrosine Phosphorylation of Eps15 by the EGFR Requires PTB of Cbl but Not Its C-terminal Region—To establish a link between the ubiquitinated EGFR and Eps15, we examined the requirements for Eps15 phosphorylation. Eps15 is known to be phosphorylated on tyrosine residues upon EGFR stimulation, possibly by the EGFR kinase domain (42). Previously, we have used Eps15 phosphorylation as evidence for communication between the EGFR and Eps15 and found that it required the ubiquitin ligase function of c-Cbl (32). Therefore, we now tested the effect of the N- and C-terminal Cbl mutants on Eps15 phosphorylation. CHO cells were transfected with EGFR, FLAG-Eps15, and HA-tagged wild type or mutant Cbl. Cells were stimulated with EGF for 0 or 10 min, and FLAG-Eps15 was immunoprecipitated. Controls show that similar amounts of FLAG-Eps15 and HA-Cbl were expressed in the different transfected populations (TCLs) and that similar amounts of FLAG-Eps15 were isolated from these (Fig. 3). As described in our previous study (32), EGFR activation in control cells expressing only endogenous c-Cbl resulted in Eps15 phosphorylation, which was enhanced by overexpression of c-Cbl (Fig. 3). In contrast, overexpression of the c-CblG306E mutant with a disrupted PTB domain did not increment Eps15 phosphorylation over control levels. The Cbl/H9004C mutant, however, enhanced Eps15 phosphorylation over control levels to the same extent as wild-type c-Cbl (Fig. 3). Interestingly, both 70Z-Cbl and v-Cbl mutants fully inhibited Eps15 phosphorylation, while 70Z-CblG306E could not override Eps15 phosphorylation. These data indicate that the requirements for Eps15 tyrosine phosphorylation following EGFR stimulation are the same as the requirements for EGFR ubiquitination: Eps15 is only phosphorylated when c-Cbl binds to the activated receptor via its PTB but not when it binds via its C-terminal region. Thus, ligand-induced ubiquitination of the EGFR and tyrosine phosphorylation of Eps15 are perfectly correlated, in line with the idea that Eps15 can come into proximity of the activated EGFR by virtue of ubiquitin modification of the receptor.

The PTB Domain and C Terminus of c-Cbl Can Independently Support Recruitment of Eps15 to the Plasma Membrane—To directly follow recruitment of Eps15 to the activated EGFR at the plasma membrane, we monitored the distribution of endogenous Eps15 in CHO cells by confocal microscopy. Cells transfected with EGFR and wild-type or mutant Cbl were incubated with TxR-labeled EGF for 1 h on ice to allow for binding to the EGFR and next for 2 min at 37 °C to allow for EGFR signaling. We confirmed our previous data (32) that ligand binding to the EGFR induced re-localization of Eps15...
from the cytoplasm to the plasma membrane (Fig. 4). This already occurred in the presence of endogenous c-Cbl (data not shown) and was increased after overexpression of c-Cbl (Fig. 4, A–D). This figure also illustrates the original finding that prompted our studies: the EGF-induced membrane localization of c-Cbl at this early time point (18). In the overlay in Fig. 4D, simultaneous co-localization of the ligand-bound EGFR, c-Cbl, and Eps15 at the plasma membrane can be observed.

To assess the requirements for Eps15 recruitment, we focused on the effects of ubiquitin ligase-deficient Cbl mutants. As reported previously (32), 70Z-Cbl completely abrogated Eps15 recruitment (Fig. 4F). Ligand-bound EGFR and 70Z-Cbl co-localized at the plasma membrane, but Eps15 was only found in the cytoplasm (Fig. 4H). Surprisingly though, despite its dominant negative effect on EGFR ubiquitination and Eps15 phosphorylation, v-Cbl did not disrupt Eps15 recruitment (Fig. 3, I–L). The 70Z-CblG306E mutant had no dominant negative effect either (Fig. 3, M–P).

Thus, blocking of either the PTB binding site on the EGFR by v-Cbl or the Grb-2 binding site by 70Z-CblG306E still allowed for recruitment of Eps15 to the activated EGFR at the plasma membrane. Blocking of both sites by 70Z-Cbl abrogated recruitment, indicating that ubiquitin ligase-proficient endogenous c-Cbl could support Eps15 recruitment via either binding mode to the EGFR. The observation that v-Cbl had a dominant negative effect on EGFR ubiquitination but not on Eps15 recruitment indicates that recruitment can proceed in the absence of EGFR ubiquitination. Since we have provided strong evidence that recruitment involves interaction between a ubiquitin-modified docking protein and the UIM of Eps15 (32), there must be an alternative for the EGFR to act as docking site.

**Interaction between c-Cbl and CIN85 Is Not Essential for Eps15 Recruitment**—CIN85 was an interesting candidate to act as alternative docking site for the UIM of Eps15, since it is mono-ubiquitinated by c-Cbl and has been implicated in c-Cbl-induced EGFR internalization (35, 37). To study a possible involvement of CIN85 in Eps15 recruitment, we used a c-Cbl point mutant, c-CblR829A, in which the CIN85 binding site in its C terminus is selectively disrupted (35) (Fig. 1B). This c-Cbl mutant retains the capacity to bind to the EGFR via either its PTB or its C terminus and can therefore act as a dominant negative for CIN85 binding to Cbl molecules that localize at plasma membrane in activated cells.

First, we studied the effect of c-CblR829A on EGFR ubiquitination and Eps15 tyrosine phosphorylation. The mutant was co-expressed in COS cells with HA-ubiquitin, cells were stimulated with EGF for 0 or 15 min, and EGFR IPs were examined. (Fig. 5A). Clearly, overexpression of c-CblR829A strongly promoted EGFR ubiquitination as compared with the control situation. Receptor stimulation also enhanced the interaction between EGFR and c-CblR829A, as visualized in the anti-HA and anti-EGF immunoblots. To examine Eps15 phosphorylation, CHO cells were co-transfected with EGFR, FLAG-Eps15, and HA-c-CblR829A or control vector. Cells were stimulated with EGF for 0 and 10 min, and FLAG-Eps15 IPs were examined (Fig. 5B). Overexpression of c-CblR829A strongly enhanced EGF-induced Eps15 phosphorylation as compared with the control. Immunoblotting of total cell lysates showed similar expression levels of FLAG-Eps15 and EGFR and similar EGF activation in control and c-CblR829A transfected cells. We conclude that CIN85 binding to c-Cbl is not required for EGFR ubiquitination or Eps15 phosphorylation.

Next, we examined the effect of c-CblR829A on EGF-induced recruitment of endogenous Eps15. CHO cells were co-transfected with EGFR and HA-c-CblR829A or control vector. They were incubated with TrxR-labeled EGF for 1 h on ice, followed by stimulation for 0 or 2 min at 37 °C prior to fixation. Without stimulation, ligand-bound EGFR were present at the cell surface (Fig. 5C), and endogenous Eps15 was found throughout cytosol and nucleus and in proximity of the plasma membrane to the same extent in both transfected and untransfected cells (compare green and multicolored cells in Fig. 5F). c-CblR829A was present throughout the cytosol and to some degree at the plasma membrane (Fig. 5E), where it co-localized with activated EGFR but not with Eps15 (Fig. 5F; no white signal). After 2 min of stimulation, plasma membrane localization of Eps15 (Fig. 5H) and c-CblR829A (Fig. 5I) was increased in transfected, but not in untransfected cells, both frequently co-localizing with activated EGFR (Fig. 5J; white clusters). Together, these data show that interaction between c-Cbl and CIN85 is not essential for either Eps15 recruitment to the plasma membrane or for its phosphorylation.

**c-Cbl Can Promote EGF Internalization Independent of EGFR Ubiquitination or Eps15 Tyrosine Phosphorylation**—Our results thus far indicate that c-Cbl can recruit Eps15 to the plasma membrane upon EGFR stimulation by two distinct mechanisms: one requires the PTB of c-Cbl, but not its C-terminal region, and is accompanied by EGFR ubiquitination and Eps15 tyrosine phosphorylation. The other requires the C-terminal region of c-Cbl, but not its PTB, and can proceed in absence of EGFR ubiquitination and Eps15 tyrosine phosphorylation. Subsequent studies were performed to address which pathway was required for EGF internalization. CHO cells transfected with EGFR were used for internalization studies, since we have used these cells previously to characterize the endocytic pathway of the EGFR (32). The uptake of 125I-labeled EGF in short term assays (up to 8 min) was used as read-out for early endocytic events.
Overexpression of wild-type c-Cbl enhanced EGFR internalization, as compared with the endogenous situation (Fig. 6, control). 70Z-Cbl, however, reduced EGFR endocytosis to levels below those observed in control cells, indicating its dominant negative effect on endogenous Cbl. Expression levels of the EGFR and transfected Cbl molecules were comparable between different cell populations (results not shown). The dominant negative 70Z-Cbl did not fully abrogate EGFR endocytosis, indicating that a Cbl-independent pathway exists to remove EGFR from the cell surface. We have shown previously that this residual pathway is fully clathrin-dependent (by K⁺ depletion) but is devoid of Eps15 (32). Importantly, v-Cbl did not promote EGFR endocytosis over control levels, in contrast to c-Cbl. However, it also did not have a dominant negative effect, in contrast to 70Z-Cbl. Since v-Cbl completely overruled EGFR ubiquitination (Fig. 2) and Eps15 tyrosine phosphorylation (Fig. 3) by endogenous c-Cbl, we can conclude that these events are not required to allow c-Cbl to promote EGFR internalization. Rather, its capacity to promote removal of EGFR from the cell surface is strictly correlated with its capacity to recruit Eps15 to the plasma membrane after EGFR stimulation (Fig. 4). Thus, by means of its ubiquitin ligase activity, c-Cbl can promote EGFR endocytosis via two independent binding modes, one requiring its N terminus (lost in presence of v-Cbl) and one requiring its C terminus (retained in presence of v-Cbl). Both of these involve Eps15 recruitment.

**DISCUSSION**

The work reported here was inspired by our original observation that c-Cbl associates with and ubiquinates the EGFR prior to endocytosis. Electron microscopy of EGFR-stimulated cells showed co-localization of c-Cbl with EGFR at the plasma membrane, both outside and at CCP. Moreover, c-Cbl and EGFR remained associated throughout the clathrin-mediated endocytic route, since they co-localized in clathrin-coated vesicles, as well as in multivesicular bodies (18). These observations are consistent with a role for c-Cbl in receptor endocytosis, as well as in receptor sorting in endosomal compartments.

Important signaling roles for ubiquitin in both these processes have emerged and thus tie in with the role of Cbl proteins as ubiquitin ligases. However, until recently, mechanisms explaining the link between Cbl-mediated ubiquitination and receptor endocytosis or sorting were lacking.

It is unambiguous that the c-Cbl ubiquitin ligase promotes post-endocytic sorting of the EGFR toward lysosomes and thereby its degradation (5, 43, 44). Studies in both yeast and mammalian cells indicate that ubiquitin modification regulates this process. The ubiquitin-binding protein Hrs/Vps27p sorts ubiquitinated cargo toward multivesicular bodies (45–48). Subsequently, it recruits the ESCRT-1 complex that regulates multivesicular bodies sorting (49–51). It will be of interest to determine a possible role of the ubiquitin ligase activity of Cbl in these processes and in particular its targets.

Multiple studies now support an additional role for c-Cbl, which is exerted at the cell surface. Recently, two groups confirmed our finding (18) that upon EGFR stimulation, c-Cbl is recruited to the plasma membrane, where it is enriched at the rim of CCP (52, 53). The hSpry2 protein, which can negatively regulate the ubiquitin ligase function of c-Cbl by displacing Ubc from its Ring finger (54–57), was found at the rim of CCP as well. It appeared to prevent recruitment of activated EGFR from the rim deeper into the pit (52). Consistent with this, 70Z-Cbl was found to inhibit EGFR endocytosis by retaining it in CCP (53). Our recent data (32) extend these findings by showing that the ubiquitin ligase function of c-Cbl is required for EGF-induced recruitment of Eps15 to the plasma membrane via its UIM. Eps15 is expected to aid in the assembly of clathrin and other structural and regulatory components involved in formation of CCP (1). Indeed, we could show that c-Cbl-dependent Eps15 recruitment mediated entry of activated EGFR into clathrin-coated pits and vesicles characterized by the presence of Eps15 (32) (Fig. 1A).

c-Cbl can down-regulate the EGFR by two distinct mechanisms: one involving recruitment via its PTB to Tyr(P)1045 in the cytoplasmic domain of the EGFR (the direct pathway) and

**Fig. 4. Effect of ubiquitin ligase-deficient Cbl mutants on EGF-induced recruitment of Eps15 to the plasma membrane.** CHO cells co-expressing wild-type EGFR and HA-tagged c-Cbl (A–D), 70Z-Cbl (E–H), v-Cbl (I–L) or 70Z-CblG306E (M–P) were incubated with EGF-TxR (red) on ice for 1 h (A, E, I, and M). After stimulation at 37 °C for 2 min, cells were fixed and stained with anti-Eps15 pAb and fluorescein isothiocyanate-conjugated secondary antibody to detect Eps15 proteins (green) (B, F, J, and N) and with anti-HA mAb and Cy5-conjugated secondary antibody to detect Cbl proteins (blue) (C, G, K, and O). Cells were analyzed by confocal microscopy. The displayed confocal planes are from the basal half of the cells.
another involving its recruitment to other tyrosine-phosphorylated sites via Grb-2 (the indirect pathway) (36). The same study also showed that direct binding of c-Cbl to the Tyr(P)1045 allows for ubiquitination of the EGFR, while indirect binding does not favor this. Our data are consistent with this; in the presence of v-Cbl, endogenous c-Cbl can still bind to the EGFR via Grb-2, but this did not support receptor ubiquitination. Accordingly, c-CblG306E, which can only bind via Grb-2, did not enhance EGFR ubiquitination, while CblΔC, which can only bind via the PTB, did. Nevertheless, some degree of EGFR-Y1045F ubiquitination was observed upon overexpression of Grb-2 (36), while dominant negative Grb-2 could reduce EGFR ubiquitination (52). This indicates that EGFR ubiquitination can occur via the indirect pathway, but it is apparently much less efficient than via the direct pathway.

Since in our study v-Cbl completely inhibited EGFR ubiquitination, we could determine the importance of this event for Eps15 recruitment. We found that one pathway exists in which Eps15 is recruited toward the ubiquitinated EGFR at the plasma membrane. The question is whether Eps15 directly binds to a ubiquitin moiety on the activated EGFR. Whereas co-immunoprecipitation of Eps15 and the EGFR has been reported by some investigators (24, 58), we and others (24, 42, 59) could not find evidence for a direct interaction. Whether direct interaction between ubiquitin moieties on the EGFR and Eps15 is required cannot be addressed by point mutation of the ubiquitination sites in the EGFR, because they are presently undefined. They are probably localized within the kinase domain, since none of the lysine residues outside this domain are target for ubiquitination (21).

![FIG. 5. Role of the interaction between c-Cbl and CIN85 in EGF-induced Eps15 phosphorylation and plasma membrane recruitment.](image)

2 A. A. de Melker, G. van der Horst, and J. Borst, unpublished data.
ubiquitinated. Binding of Eps15 to a ubiquitin moiety in the kinase domain possibly positions it favorably to be phosphorylated by the EGFR.

Importantly, our study has revealed an alternative pathway for c-Cbl-mediated Eps15 recruitment and EGFR endocytosis, which proceeds in the absence of EGFR ubiquitination. This pathway results from indirect binding of c-Cbl to the EGFR. It implicates the involvement of a protein, other than the EGFR, that becomes ubiquitinated by c-Cbl. We explored the possibility that CIN85 is involved in Eps15 recruitment, since CIN85 binds to a C-terminal sequence and becomes mono-ubiquitinated by c-Cbl (35, 38). Our collective data exclude that the UIM of Eps15 uniquely binds to either the ubiquitinated EGFR or ubiquitinated CIN85. It is likely that the UIM of Eps15 can bind to either, depending on its mode of interaction with the EGFR. Possibly even other docking sites, ubiquitinated by c-Cbl or other ubiquitin ligases, play a role, thus enforcing the protein network necessary for proper clathrin-mediated EGFR internalization.

It has been found that a phosphorylation-negative Eps15 mutant strongly inhibited internalization of the EGFR (60). However, we have shown here that tyrosine phosphorylation of Eps15 is not required for its recruitment to the plasma membrane and also not for c-Cbl-mediated EGFR endocytosis via a clathrin-dependent pathway. In the presence of 70Z-Cbl, the Eps15 pathway was abrogated. Nevertheless, we found that EGFR internalization could still occur via a pathway that is clathrin-dependent, according to previous K⁺ depletion experiments (32), indicating the possibility of Eps15-independent clathrin-mediated internalization. Our results are supported by Belleudi et al. (61), who recently reported that the clathrin-mediated uptake of ligand-bound keratocyte growth factor receptors is not mediated by Eps15. This may also explain why the EGFR internalization rate was not affected in c-Cbl⁻⁻/⁻ mouse embryonic fibroblasts or in Chinese hamster lung cells with a temperature-sensitive defect in ubiquitination (44).

Apart from the Sprotty (Spry) proteins, which interfere with the ubiquitin ligase activity of Cbl, two newly defined c-Cbl interacting proteins, Sts-1 and Sts-2/TULA, have been shown to inhibit endocytosis and degradation of EGFR. Both of these proteins contain a ubiquitination-associated domain, highlighting the importance of ubiquitin as signaling module in the receptor internalization process (62, 63). Results of Kowanetz et al. (63) suggest that Sts-1/2 is recruited to the activated EGFR via its interaction with c-Cbl and subsequently binds with its ubiquitination-associated domain to the ubiquitinated EGFR, thus competing with a number of endocytosis-promoting proteins that contain ubiquitin binding modules, such as epsin or Eps15. This model ties in with the mechanism of c-Cbl action we have revealed, and it will be of interest to subject it to further experimental validation.

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