c-Cbl directs EGF receptors into an endocytic pathway that involves the ubiquitin-interacting motif of Eps15

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Summary

c-Cbl associates with the activated EGF receptor before endocytosis. We here reveal that the capacity of c-Cbl to promote receptor internalization depends on its ubiquitin ligase activity, which functionally connects the EGF receptor to Eps15, a mediator of clathrin-coated pit formation. EGF-induced phosphorylation of Eps15, as well as recruitment of Eps15 to the plasma membrane and its co-localization with the EGF receptor in endosomes required the ubiquitin ligase activity of c-Cbl. This suggested that ubiquitin provides a direct or indirect link between the receptor and Eps15. Indeed, EGF-induced redistribution of Eps15 to the plasma membrane and endosomes depended on its ubiquitin-interacting motif. Upon over-expression, the ubiquitin-interacting motif abrogated the capacity of c-Cbl to promote EGF receptor endocytosis and only allowed receptor internalization via a route that lacked Eps15. Our findings disclose a novel function for the c-Cbl ubiquitin ligase and identify ubiquitin as a module that directs the EGF receptor into an endocytic pathway involving Eps15.

Key words: Ubiquitin-interacting motif, EGF receptor, c-Cbl, Eps15, Endocytosis

Introduction

Ligand-induced internalization of growth factor receptor tyrosine kinases (RTK) is required for certain signalling events, but ultimately attenuates signalling due to receptor degradation in lysosomes (Sorkin and Von Zastrow, 2002). The importance of RTK downregulation is highlighted by the malignant transformation of epithelial and neuronal cells in humans as a result of constitutive over-expression of members of the ErbB family of RTK, which includes the EGF receptor (EGFR) (Waterman and Yarden, 2001). Receptors are removed from the cell surface by endocytosis. Either constitutively or upon ligand binding, they cluster in plasma membrane specializations, which subsequently invaginate and pinch off to form endosomes. Clathrin-coated pits (CCP) are specialized membrane structures, which contain the necessary signalling and structural proteins to form the starting point of clathrin-mediated endocytosis (Marsh and McMahon, 1999). Growth factor receptors, such as the EGFR, are internalized via CCP, but how they are recruited into these structures is currently unclear.

Genetic studies in *Caenorhabditis elegans* (Yoon et al., 1995), *Drosophila melanogaster* (Meisner et al., 1997) and mice (Murphy et al., 1998) have revealed that the Cbl family of multi-adaptor proteins downregulates RTK signalling. Cbl proteins share a unique phosphotyrosine (PY)-binding domain, which allows their activation-dependent association with a great variety of tyrosine kinase-based receptor systems. They also share a Ring finger domain, which binds ubiquitin-conjugating enzymes and defines them as a novel type of E3 ubiquitin ligase (Joazeiro et al., 1999; Levkowitz et al., 1999; Yokouchi et al., 1999). Some Cbl orthologues, including mammalian c-Cbl and Cbl-b, have a C-terminal region with proline-rich SH3 domain-binding sequences and tyrosine phosphorylation sites, which serve additional roles (Tsygankov et al., 2001; Smit and Borst, 1997).

Transfection studies in mammalian cells have revealed that Cbl proteins, exemplified by c-Cbl, increase ligand-induced receptor ubiquitination and downregulate RTK function by promoting removal of receptors from the cell surface, as well as their degradation (Lee et al., 1999; Levkowitz et al., 1998; Miyake et al., 1998). The prevailing thought is that c-Cbl plays a role post-endocytosis. It has been shown to interfere with receptor recycling towards the plasma membrane by promoting endosomal sorting of RTK towards lysosomes (Levkowitz et al., 1998; Longva et al., 2002). However, we have previously shown that c-Cbl already associates with the EGFR in CCP at the plasma membrane, before scission of endocytic vesicles, and remains associated throughout the endocytic route (de Melker et al., 2001). A function for c-Cbl at the plasma membrane was also suggested by studies on CSF-1 receptor routing in c-Cbl deficient macrophages (Lee et al., 1999). Furthermore, the finding that the C-terminus of c-Cbl recruits endophilin, an enzyme with fatty acid transferase activity, suggests a role in vesicle budding from the plasma membrane (Petrelli et al., 2002; Soubeyran et al., 2002).

Does the ubiquitin ligase function of c-Cbl play a role in early endocytic events? A number of observations support this idea. It has been demonstrated that the EGFR is ubiquitinated

before endocytosis (de Melker et al., 2001; Stang et al., 2000). In yeast, ubiquitin has been shown to carry an internalization signal (Shih et al., 2000) and mono-ubiquitination of several classes of plasma membrane receptors enhances ligand-dependent receptor internalization (Hicke, 2001; Terrell et al., 1998). Recently, it was found that the EGFR becomes mono-ubiquitinated at multiple sites upon activation (Haglund et al., 2003a; Haglund et al., 2003b; Mosesson et al., 2003). The identification of several classes of ubiquitin-binding domains explained how mono-ubiquitin can perform its signalling role (Di Fiore et al., 2003; Hicke and Dunn, 2003). Eps15, Eps15R and Epsins, components of the CCP, all contain ubiquitin-interacting motifs (UIM) (Hofmann and Falquet, 2001; Shih et al., 2002), which suggests that they may act as ubiquitin receptors during early steps in clathrin-mediated endocytosis.

Eps15 was originally identified as a substrate of the EGFR kinase pathway (Fazioli et al., 1993) and subsequently implicated in endocytosis (Benmerah et al., 1998; Benmerah et al., 2000; Carbone et al., 1997). Eps15 has multiple interaction domains. Three copies of the EH domain in its N-terminus allow it to bind to endocytic and sorting proteins, such as Epsin (Chen et al., 1998). Eps15 is recruited to the plasma membrane upon EGFR stimulation (Torrisi et al., 1999) and can localize to coated pits (Tebar et al., 1996). Via a domain in its C-terminus, it constitutively interacts with AP-2. Eps15 mutants and antibodies to Eps15 inhibited EGF uptake, suggesting an important role of Eps15 in EGFR internalization (Carbone et al., 1997). Additional studies showed that Eps15 is involved in clathrin coat assembly, possibly by targeting AP-2 to the plasma membrane (Benmerah et al., 1998; Benmerah et al., 2000). Eps15 has two UIM, of which the first is required for EGF-induced mono-ubiquitination of Eps15 itself, whereas the second UIM in addition can bind ubiquitin (Polo et al., 2002; Klapisz et al., 2002).

In this paper, we demonstrate that c-Cbl promotes the removal of activated EGFR from the plasma membrane by directing the EGFR into an endocytic pathway that involves Eps15. This requires the ubiquitin ligase activity of c-Cbl and the UIM of Eps15. We suggest that the ubiquitinated EGFR or another c-Cbl substrate that is ubiquitinated upon EGFR activation recruits Eps15 to the plasma membrane via its UIM. This event would facilitate EGFR internalization via a clathrin-dependent route in which Eps15 plays a role.

Materials and Methods

Cell culture and antibodies

Chinese hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal calf serum, non-essential amino acids and antibiotics. Antibodies used were: rabbit polyclonal serum 282.7 directed against the EGFR (for blotting; L. H. Defize, Hubrecht Laboratory, Utrecht, The Netherlands); the mouse 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 fluorescence; Roche); mAb M2 directed against the FLAG-tag (Sigma); rabbit pAb H-896 directed against Eps15 (Santa Cruz Biotechnology); rabbit pAb C-15, directed against c-Cbl (Santa Cruz Biotechnology); mAb 4G10 against PY (Upstate Biotechnology, Lake Placid, NY). For enhanced chemiluminescence, horseradish peroxidase-conjugated rabbit anti-mouse or swine anti-rabbit Ig (DAKO, Glostrup, Denmark) were used. For immunofluorescence, affinity purified FITC-conjugated goat anti-mouse- or anti-rabbit IgG and Cy5-conjugated goat anti-rat IgG (all from Rockland, Gilbertsville, PA) were used.

Constructs

The pMT2 expression vectors containing cDNA encoding the EGFR or HA-tagged c-Cbl have been described previously (de Melker et al., 2001). The HA-tagged 70Z-Cbl construct was made by PCR, using pGEM 4Z-c-Cbl (a gift from W.Y. Langdon, University of Western Australia, Australia) as a template, and cloned into pMT2HA. The Y1045F point mutation was introduced into the wild-type EGFR using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. The FLAG-tagged wild-type Eps15 cDNA (Eps15-WT) and the mutant Eps15-L883A/L885A (Eps15-UIM$^-$) in pMT2SM were a gift from E. Klapisz and P. van Bergen en Henegouwen (University of Utrecht, The Netherlands). FLAG-tagged UIM$^-$WT and UIM$^-$mut are PCR derivates of pMT2-FLAG-Eps15-WT and pMT2-FLAG-Eps15-UIM$^-$, respectively, encoding for amino acids 741-897. Fragments were cloned *Bam*HI-*Xba*I into pMT2-FLAG-Eps15-WT, thus replacing the encoding sequences of Eps15-WT, but leaving the FLAG-tag in place. Correct cloning of the mutants was verified by sequencing.

Transfections

CHO cells (3×10^5 per 10 cm^2 well) were transfected using lipofectamine PLUS Reagent (Gibco), as described previously (de Melker et al., 2001). For immunofluorescence, cells were transfected using Fugene (Roche, Indianapolis, IN) according to the manufacturer's instructions. Per 10 cm^2 well, a total of 2 μg DNA was used in a ratio of 1:6 to the Fugene solution.

Receptor internalization assay

Mouse EGF (Becton Dickinson Labware, Bedford, MA) was labelled with ^{125}I using the chloramine T method to a specific activity of about 1×10^5 cpm/ng. Transfected cells were plated in 24-well plates and allowed to attach for 24 hours. Cells were cultured in medium without serum (DMEM, 20 mM HEPES pH 7.4, 0.1% BSA (DHB)) for 3 hours. To determine the total amount of receptors present, cells were incubated with ^{125}I-EGF for 90 minutes on ice. To measure ligand internalization, cells were incubated with 1 ng/ml ^{125}I-EGF, in a total volume of 0.5 ml, for 0, 2, 4 and 8 minutes at 37°C. Cells were put on ice to stop further internalization and washed twice with PBS to remove unbound ligand. Subsequently, cells were incubated twice for 2 minutes with PBS/HCl pH 2.1. Samples were collected, after which cells were lysed in 1 M NaOH for 10 minutes. Acid wash samples representing the membrane bound ligand fractions and cell lysates representing the internalized ligand fractions were counted in a γ-counter. All experiments contained triplicate samples, except the experiment presented in Fig. 1F, which contained duplicates. Clathrin-mediated internalization was blocked using the K^+ depletion method exactly as described (Hansen et al., 1993).

Immunoprecipitation and immunoblotting

Cells were used for immunoprecipitation 48 hours after transfection. Cells were serum starved for 3 hours and stimulated with 25 ng/ml EGF (Becton Dickinson Labware, Bedford, MA) at 37°C. After stimulation, cells were put on ice, washed with cold PBS and incubated for 30 minutes at 0°C with lysis buffer (30 mM Tris-HCl pH 7.5, 1% NP-40, 150 mM NaCl, 4 mM EDTA pH 8.0, 10 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml phenylmethylsulfonyl fluoride, 0.1 μM leupeptin and 0.1 μM aprotinin). Cell lysates were clarified by centrifugation for 10 minutes at 13,000 ***g***. Immunoprecipitation and immunoblotting were performed as

described (de Melker et al., 2001). Proteins were visualised using enhanced chemiluminescence (Amersham Pharmacia Biotech, Roosendaal, The Netherlands).

Immunofluorescence

One day after transfection, cells were detached with trypsin/EDTA and plated on cover slips placed in 6-well plates. Cells were allowed to attach for 24 hours and then serum starved for 3 hours in DMEM containing 20 mM HEPES and 0.1% BSA. After 10 minutes temperature adjustment of the cells on ice, TxR-labelled EGF (100 ng/ml; Molecular Probes, Eugene, OR) was allowed to bind for 1 hour on ice. Cells were stimulated at 37°C and fixed with methanol kept at –20°C. Cells were rehydrated in PBS, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ for 5 minutes and blocked for 30 minutes using 1% BSA in PBS, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Incubations were performed with antibodies diluted in blocking buffer. Cover slips were mounted in Vectashield (Vector Laboratories, Burlingham, CA) and viewed under a Leica TCS NT confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany). To minimize spectral leak-through, three colour images were obtained by sequential scanning. Only cells expressing low levels of FLAG-Eps15 were analyzed.

Results

c-Cbl stimulates EGFR internalization via its ubiquitin ligase activity

We established previously that the activated EGFR recruits c-Cbl and is ubiquitinated by it at the plasma membrane, before its entry into clathrin-coated vesicles (de Melker et al., 2001). The aim of the work reported here was to establish whether the ubiquitin ligase function of c-Cbl plays a role in recruitment of EGFR into the clathrin-mediated internalization route. To investigate this, we made use of a c-Cbl mutant that selectively lacks ubiquitin ligase activity (Fig. 1A). The 70Z-Cbl mutant has a deletion of 17 amino acids, which disrupts the Ring finger structure and thereby the capacity to bind ubiquitin-conjugating enzymes (Yokouchi et al., 1999). Like wild-type Cbl molecules, the 70Z-Cbl mutant can directly bind to phosphorylated Y1045 in the cytoplasmic tail of the activated EGFR, via its PY-binding domain (Levkowitz et al., 1999; Waterman et al., 2002). Other interaction motifs, such as the

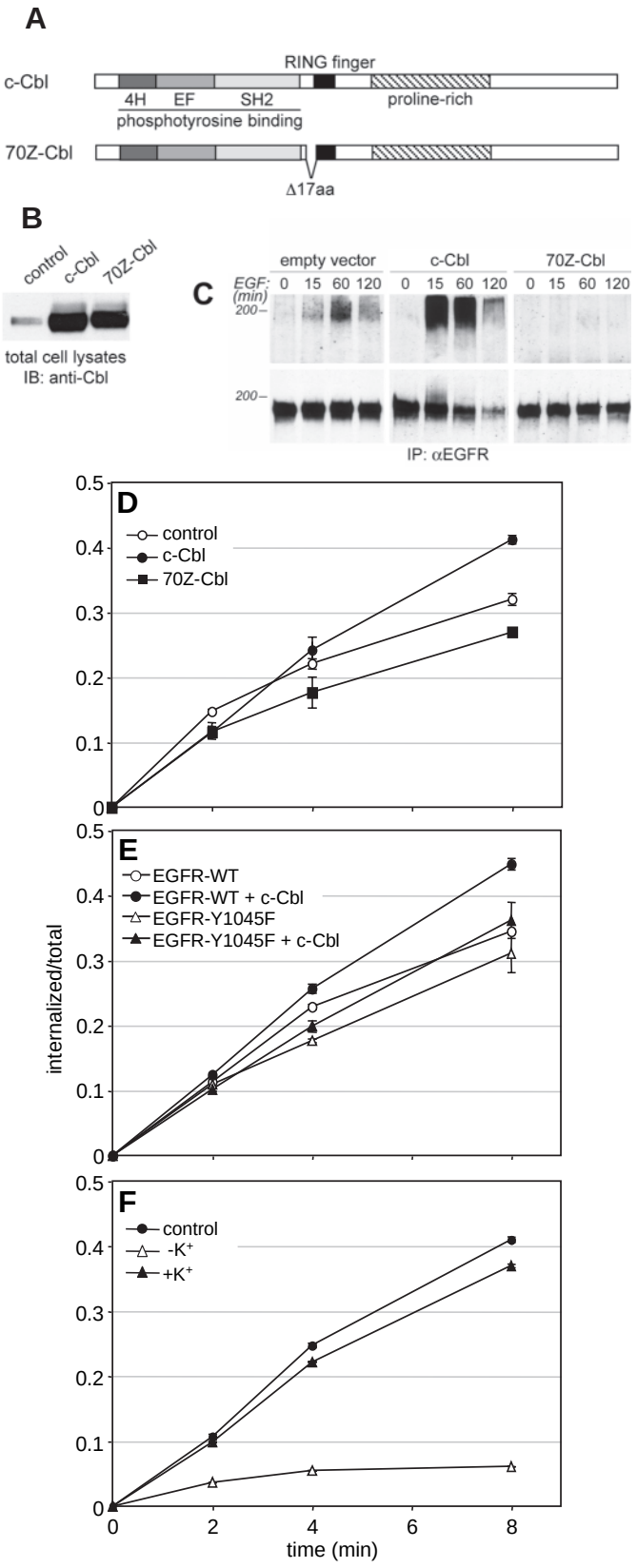

Fig. 1. Ubiquitin ligase activity of c-Cbl contributes to clathrin-mediated EGFR internalization. (A) Wild-type c-Cbl and 70Z-Cbl proteins used in this study. 70Z-Cbl lacks 17 amino acids including part of the Ring finger domain. (B) CHO cells were transfected with empty vector, c-Cbl or 70Z-Cbl. Western blots of total cell lysates were probed with antibodies against c-Cbl. (C) CHO cells were co-transfected with EGFR, HA-ubiquitin and empty vector, c-Cbl or 70Z-Cbl. Anti-EGFR immunoprecipitates from EGF-stimulated cells were immunoblotted with anti-HA mAb to detect ubiquitinated EGFR and pAb to the EGFR to detect total EGFR protein amounts. (D) CHO cells co-expressing the EGFR and empty vector, c-Cbl or 70Z-Cbl were incubated at 37°C with 1 ng/ml ^{125}I-labelled EGF. Internalization was monitored as outlined in Materials and Methods. Presented is the ratio of internalized to total-cell-associated radioactivity. Expression controls of the Cbl proteins are shown in B. (E) CHO cells expressing wild-type EGFR (EGFR-WT) or EGFR-Y1045 in the presence or absence of exogenous c-Cbl were used in an internalization assay as described in D. (F) CHO cells were co-transfected with full length c-Cbl and wild-type EGFR and treated as described in D, using 20 ng/ml radiolabelled EGF (control), or subjected to hypotonic shock followed by incubation with ligand in the absence (○) or presence (●) of K^+ ions. Experiments in D and E contained triplicate samples, error bars represent standard deviations. In F, both values of duplicate samples are shown and means are connected by a line.

proline-rich region and several tyrosine residues in the C-terminus are also unaltered in 70Z-Cbl compared with c-Cbl (Andoniou et al., 1994).

We first tested the effect of 70Z-Cbl on EFGR ubiquitination. CHO cells were used, because they have no endogenous EGFR (Tzahar et al., 1996) and can therefore be reconstituted with EGFR or mutants thereof. Moreover, EGFR internalization is efficient in these cells and they are frequently used as a model to study endocytic pathways of the EGFR (Levkowitz et al., 1998; Soubeyran et al., 2002). We have previously demonstrated that in these cells, c-Cbl and the EGFR co-localize after EGFR activation at the plasma membrane and in clathrin-coated vesicles (de Melker et al., 2001). CHO cells contain low levels of endogenous c-Cbl (Fig. 1B) and the effects of c-Cbl can be significantly enhanced by its transient over-expression. In the absence of c-Cbl transfection, the EGFR was ubiquitinated in response to EGF stimulation, in a transient manner, with signals peaking at about 60 minutes (Fig. 1C). Increased c-Cbl expression accomplished by transfection strongly enhanced both kinetics and levels of ligand-induced EGFR ubiquitination. Importantly, the 70Z-Cbl Ring finger mutant completely inhibited EGF-induced receptor ubiquitination. It exerted a dominant negative effect on endogenous Cbl, because the EGFR ubiquitination observed in the absence of c-Cbl transfection was also abrogated. This was expected from its unaltered EGFR binding capacity, which allows 70Z-Cbl to compete for EGFR binding with endogenous Cbl molecules. The efficiency of EGFR ubiquitination correlated with the efficiency of its downregulation, as shown by anti-EGFR immunoblotting (Fig. 1C). These data are in agreement with earlier studies (Levkowitz et al., 1998).

The uptake of ^{125}I-labelled EGF in short-term assays (up to 8 minutes) was used as a read-out for early endocytic events (Fig. 1D). Over-expression of wild-type c-Cbl enhanced EGFR internalization, compared with the endogenous situation (control). In contrast, 70Z-Cbl failed to stimulate EGFR internalization. Moreover, it reduced EGFR endocytosis to levels below those observed in control cells. This indicates that 70Z-Cbl exerted a dominant negative effect on endogenous Cbl. Expression levels of transfected c-Cbl and its mutant were comparable in these assays (Fig. 1B). Clearly, the dominant negative 70Z-Cbl did not completely abrogate EGFR endocytosis. It appears therefore, that other internalization pathways exist for the EGFR, which are Cbl independent.

Mutation of the c-Cbl binding site in the EGFR at Y1045 consistently reduced the efficiency of EGFR internalization in control cells that relied on endogenous c-Cbl (Fig. 1E). Moreover, exogenous c-Cbl was less able to stimulate endocytosis of the mutant EGFR than of the wild-type EGFR. The residual stimulation of mutant EGFR by c-Cbl is probably due to the alternative mode of c-Cbl receptor binding via Grb-2. Effects of c-Cbl and mutants on EGFR endocytosis were comparable when CHO cells were stimulated with low (1 ng/ml) or high (20 ng/ml) concentrations of EGF (results not shown). Even at 20 ng/ml EGF, EGFR internalization in these cells was fully clathrin dependent as established by K^+ depletion (Fig. 1F).

We conclude that mutation of the Ring finger domain of c-Cbl prevents EGFR ubiquitination and reduces receptor internalization. Apparently, the ubiquitin ligase function of c-Cbl can promote clathrin-dependent endocytosis of the EGFR, but is not absolutely required for it.

EGF-induced tyrosine phosphorylation of Eps15 requires the ubiquitin ligase activity of c-Cbl

If c-Cbl indeed enhances clathrin-mediated endocytosis of the EGFR via protein ubiquitination, it may regulate proteins that contain motifs capable of interaction with ubiquitin. Eps15 contains two such UIM. It has been described as a substrate of the EGFR kinase in vitro and becomes rapidly phosphorylated on tyrosine upon EGFR activation in intact cells (Fazioli et al., 1993). We determined whether EGF-induced Eps15 phosphorylation required the ubiquitin ligase function of c-Cbl. For this purpose, CHO cells were transfected with wild-type EGFR and Eps15, in the presence of wild-type c-Cbl or 70Z-Cbl. Controls depict the amount of EGFR expressed, the extent of EGFR stimulation (as determined by tyrosine phosphorylation), as well as the amount of Eps15 and Cbl expressed in the various cell populations. Although some degree of EGFR activation was observed at t=0, in particular in control and 70Z-Cbl expressing cells, this did not coincide with phosphorylation of Eps15. Eps15 tyrosine phosphorylation was contingent upon stimulation of cells with EGF. It was observed in control CHO cells, which contain endogenous c-Cbl (Fig. 2A) and more pronounced upon over-expression of c-Cbl. In contrast, in the presence of the ubiquitin-ligase-deficient 70Z-Cbl mutant, Eps15 phosphorylation was virtually abrogated, indicating the dominant negative effect of this mutant on endogenous c-Cbl function. These results indicate that communication between the EGFR and Eps15 requires c-Cbl-mediated ubiquitination of some component in the pathway, possibly the EGFR itself.

Mutation of the c-Cbl binding site at Y1045 in the EGFR affected EGF-induced Eps15 phosphorylation (Fig. 2B). While wild-type EGFR induced rapid and sustained Eps15 tyrosine phosphorylation, the capacity of the Y1045F EGFR mutant to do so was impaired, even when c-Cbl was co-expressed (Fig. 2B). These results suggest that EGF-induced phosphorylation of Eps15 depends to a large extent on binding of c-Cbl to Y1045 in the EGFR.

Co-localization of the EGFR with Eps15 at the plasma membrane and in endosomes requires the ubiquitin ligase activity of c-Cbl

Eps15 is recruited from the cytosol to the plasma membrane upon EGF stimulation (Torrisi et al., 1999). To determine whether co-localization of Eps15 and EGFR at the plasma membrane and in endosomes was dependent on the ubiquitin ligase activity of c-Cbl, we examined this in the presence of c-Cbl and the 70Z-Cbl mutant. Confocal microscopy was used to monitor the cellular distribution of transfected HA-tagged Cbl, FLAG-tagged Eps15 and the EGFR. Cells were incubated with Texas Red (TxR)-labelled EGF for 1 hour on ice and then stimulated for 1 or 5 minutes at 37°C. In c-Cbl expressing cells that had been incubated with TxR-EGF on ice (0 minutes), ligand-bound receptors were localized at the plasma membrane (Fig. 3A) and Eps15 was mainly diffusely distributed in the cytosol, with some localization at the plasma membrane (Fig. 3B). In accordance with our previous findings (de Melker et

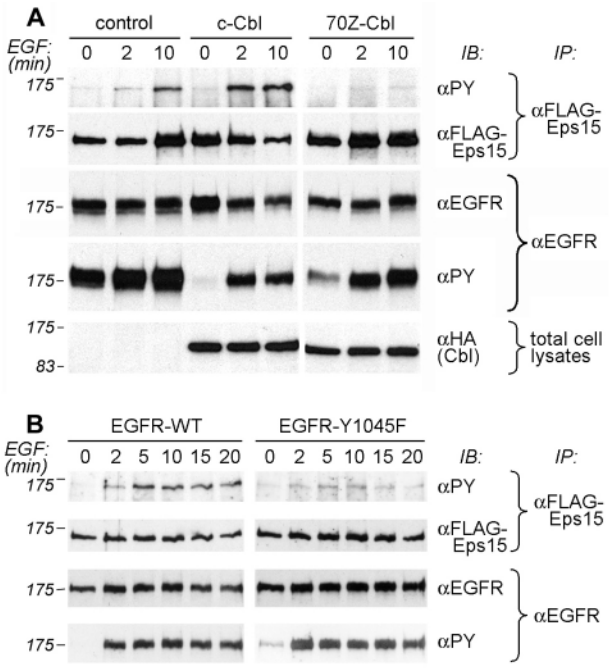

Fig. 2. EGF-induced Eps15 phosphorylation depends on the ubiquitination function of c-Cbl and Y1045 of the EGFR. (A) CHO cells co-transfected with wild-type EGFR, FLAG-tagged Eps15 and empty vector, wild-type c-Cbl, or 70Z-Cbl were stimulated with EGF. Anti-FLAG immunoprecipitates (IP) were first probed with mAb to PY, stripped and reprobed with mAb to the FLAG tag. Anti-EGFR IPs were similarly probed in succession with anti-PY and anti-EGFR mAb and total cell lysates were immunoblotted (IB) with mAb to the HA-tag, as indicated. (B) CHO cells co-transfected with full-length c-Cbl, FLAG-tagged Eps15 and EGFR-WT or EGFR-Y1045F were stimulated with EGF. Anti-FLAG and anti-EGFR immunoprecipitates were immunoblotted with antibodies to PY (PY), FLAG-tag or EGFR.

al., 2001), c-Cbl clearly co-localized with the EGFR at the plasma membrane (Fig. 3C) and in addition was diffusely distributed throughout the cytoplasm. After 1 minute of stimulation at 37°C, enhanced localization of Eps15 at the plasma membrane was visible (Fig. 3F), as well as co-localization with ligand-bound EGFR and c-Cbl at the plasma membrane, resulting in a white signal (Fig. 3H). After 5 minutes of stimulation, ligand-bound EGFR was internalized and co-localized with Eps15 and c-Cbl in vesicles (Fig. 3I-L). In control cells in which c-Cbl was not over-expressed, effects of EGFR activation on Eps15 localization were similar, indicating that endogenous c-Cbl is capable of mediating the same effects (not shown).

Like c-Cbl, the 70Z-Cbl mutant co-localized with ligand-bound EGFR at the plasma membrane after 1 hour incubation on ice (Fig. 3O,P). Diffuse staining of 70Z-Cbl throughout the cytoplasm was less frequently observed, possibly due to its efficient association with the activated EGFR at the plasma membrane. Importantly, localization of Eps15 at the plasma membrane after 1 hour of incubation on ice was undetectable in cells expressing 70Z-Cbl (Fig. 3N,P). Moreover, no membrane localization of Eps15 was observed after 1 minute of stimulation at 37°C (Fig. 3R,T). In the presence of 70Z-Cbl, Eps15 was only detectable throughout the cytoplasm (Fig. 3N,R). After 5 minutes of stimulation, ligand-bound EGFR were clustered at or close to the plasma membrane, with some localization in intracellular vesicles (Fig. 3U), but much less so than in presence of wild-type c-Cbl (Fig. 3I). Moreover, EGFR-containing endocytic structures did not contain Eps15 at all (Fig. 3V,X). 70Z-Cbl co-localized with the ligand-bound EGFR in endosomes (Fig. 3W,X), indicating that, like c-Cbl, it remains associated with the EGFR upon internalization. Clearly, 70Z-Cbl inhibited Eps15 recruitment to the plasma membrane and its co-localization with EGFR in endocytic compartments in a dominant negative manner.

Abrogation of the c-Cbl binding site in the EGFR at Y1045 affected its capacity to induce Eps15 relocalization. While Eps15 was recruited from the cytosol to the plasma membrane after stimulation of the wild-type EGFR (Fig. 4A-D), it remained diffusely distributed throughout the cytosol after stimulation of the Y1045F EGFR mutant (Fig. 4G-J). Accordingly, only after stimulation of the wild-type EGFR (Fig. 4E,F), but not the mutant EGFR (Fig. 4K,L), receptor and Eps15 were co-localized in endosomes.

We conclude that the ubiquitin ligase function of c-Cbl is required for EGF-induced recruitment of Eps15 to the plasma membrane, as well as internalization of the activated EGFR via an endocytic route that involves Eps15. In addition, Y1045 in the EGFR is important for these events.

EGF-induced tyrosine phosphorylation and redistribution of Eps15 requires the UIM of Eps15

Our data suggest that ubiquitin serves as a binding module to connect directly, or indirectly, the activated EGFR and Eps15. The C-terminus of Eps15 contains two UIM. Only the second motif (amino acids 878-897) allows Eps15 to bind to ubiquitin-containing proteins (Klapisz et al., 2002; Polo et al., 2002). We used Eps15 with mutations L883A/L885A (Eps15-UIM$^-$; Fig. 5A), which renders the UIM nonfunctional in ubiquitination and ubiquitin binding (Klapisz et al., 2002). First, we investigated whether these mutations disrupted the communication between the EGFR and Eps15, as evidenced by EGF-induced tyrosine phosphorylation of Eps15. CHO cells were transfected with wild-type Eps15 or the Eps15-UIM$^-$ mutant in the presence of c-Cbl. Stimulation of these cells with EGF for 10 minutes induced tyrosine phosphorylation of wild-type, but not of mutant Eps15 (Fig. 5B). We conclude that the activated EGFR cannot signal to Eps15 when this molecule is lacking an intact UIM.

Second, we examined the impact of the UIM mutation on the co-localization of the ligand bound EGFR with Eps15 at the plasma membrane and in endosomes. Confocal microscopy revealed a clear difference in EGF-induced re-distribution of the Eps15-UIM$^-$ mutant versus wild-type Eps15. After incubation of cells with EGF-TxR for 1 hour on ice, membrane localization of wild-type or mutant Eps15 was infrequent (Fig. 5C,D,I,J). After 1 minute of stimulation at 37°C, wild-type Eps15 re-distributed from cytosol to plasma membrane (Fig. 5E), but the Eps15-UIM$^-$ mutant did not (Fig. 5K). Consequently, the Eps15 mutant did not co-localize with ligand-bound EGFR at the plasma membrane (compare Fig. 5F,L). After 5 minutes of stimulation, wild-type Eps15 co-localized with EGFR in endosomes (Fig. 5G,H). In contrast,

the Eps15-UIM$^-$ mutant remained diffusely distributed throughout the cytosol (Fig. 5M) and was not found at all in the endocytic vesicles that contained the internalized EGFR (Fig. 5N). These results indicate that EGF-induced recruitment of Eps15 towards the plasma membrane and its subsequent co-localization with the EGFR in endocytic structures requires a functional UIM in Eps15. These data argue that binding of this UIM to a ubiquitin moiety is necessary to connect, functionally and physically, the activated EGFR and Eps15.

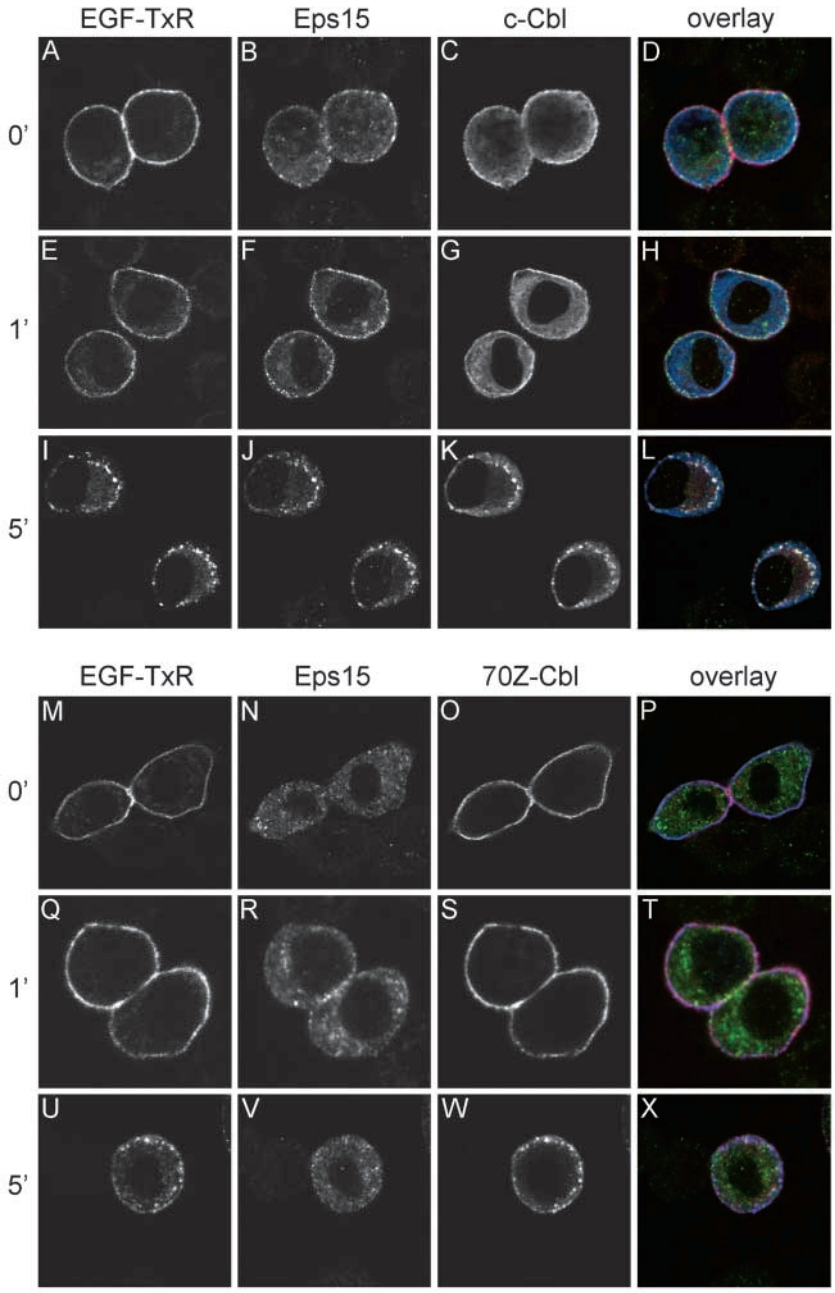

Fig. 3. Ubiquitin ligase activity of c-Cbl determines EGF-induced recruitment of Eps15 towards plasma membrane and endosomes. CHO cells co-expressing FLAG-Eps15, wild-type EGFR and HA-c-Cbl (A-L) or HA-70Z-Cbl (M-X) were incubated with EGF-TxR (red) (A,E,I,M,Q,U) on ice for 1 hour. After stimulation at 37°C for 0 minutes (A-D,M-P), 1 minute (E-H,Q-T) or 5 minutes (I-L,U-X), cells were fixed and stained with anti-FLAG mAb and FITC-conjugated secondary antibody to detect Eps15 proteins (green) (B,F,J,N,R,V) and with anti-HA mAb and Cy5-conjugated secondary antibody to detect Cbl proteins (blue) (C,G,K,O,S,W). Cells were analyzed by confocal microscopy. The displayed confocal planes are from the basal half of the cells.

Over-expression of the UIM of Eps15 abrogates the capacity of c-Cbl to promote EGFR endocytosis

Next, we attempted to assess the effect of wild-type Eps15 versus the Eps15-UIM$^-$ mutant on EGFR internalization. However, over-expression of wild-type Eps15 inhibited EGFR endocytosis (results not shown). Eps15 can bind to a number of proteins involved in clathrin-mediated endocytosis, such as AP-2, epsin and synaptojanin. Possibly, its over-expression sequesters endogenous pools of such proteins and thus disrupts receptor internalization.

Therefore, we used another approach to provide further evidence for the possibility that the UIM of Eps15 is required to connect the activated EGFR and Eps15. We constructed a truncated form of Eps15 that contains both UIM, but no other defined domains (UIM-WT, Fig. 5A), with the anticipation that it behaves similarly with respect to ubiquitin binding as full-length Eps15, but exerts a dominant negative effect on c-Cbl-mediated EGFR endocytosis. As a control, the mutations L883A/L885A were introduced in the second UIM of this construct (UIM-mut, Fig. 5A). Interestingly, the capacity of c-Cbl to promote EGFR internalization was fully abrogated by over-expression of UIM-WT (Fig. 6A). In contrast, UIM-mut did not have such a dominant negative effect on c-Cbl-mediated endocytosis (Fig. 6B). These data support the concept that c-Cbl promotes EGFR endocytosis by establishing a ubiquitin-UIM interaction.

Over-expression of the UIM of Eps15 abrogates plasma membrane recruitment of Eps15 and its participation in EGFR endocytosis

To examine whether UIM-WT prevented the participation of endogenous Eps15 in the EGFR internalization route, we performed confocal microscopy. First, we established that EGFR stimulation modulated the cellular localization of endogenous Eps15 in a similar way to that of the exogenous FLAG-tagged Eps15, which was used previously as a read-out. In control cells transfected with EGFR only, which had been incubated with TxR-labelled EGF for 1 hour on ice, endogenous Eps15 was present in a punctate pattern at or near the plasma membrane and in the cytosol. Occasional co-localization of endogenous

Eps15 with ligand-bound EGFR at the plasma membrane was visible (not shown). After 1 minute of stimulation at 37°C, plasma membrane localization of Eps15 in EGFR-expressing cells was strongly enhanced compared with untransfected cells (Fig. 7B) and a clear co-localization with ligand-bound receptors could be detected (Fig. 7C). After 5 minutes of incubation at 37°C, EGFR were internalized and localized in cytoplasmic vesicles. Co-localization of endogenous Eps15 and ligand-bound EGFR in these endosomal structures was somewhat less pronounced than we had found earlier for FLAG-tagged Eps15 (Fig. 3C) and was mainly observed close to the plasma membrane (Fig. 7D). We conclude that endogenous Eps15, like exogenous Eps15 is efficiently recruited from the cytosol to the plasma membrane upon EGFR stimulation. The greater abundance of exogenous Eps15 in endosomes after EGF stimulation can be explained by the larger cytoplasmic pool created by transfection.

In the presence of UIM-WT, Eps15 was not recruited to the plasma membrane upon 1 minute of EGF stimulation (Fig. 7F). Moreover, no co-localization of Eps15 and ligand-bound EGFR could be observed (Fig. 7G). At 5 minutes after EGFR activation, Eps15 did not localize to endosomes containing EGFR, also not in those close to the plasma membrane, while the EGFR itself was still internalized (Fig. 7H). To determine whether EGF-induced Eps15 re-distribution depended on an intact second UIM, we over-expressed EGFR together with UIM-mut. Indeed, in cells expressing UIM-mut, EGF-induced re-distribution of Eps15 occurred as in the control situation. We found strong expression of Eps15 at the plasma membrane at 1 minute after stimulation (Fig. 7J), where it co-localized with ligand-bound EGFR (Fig. 7I-K). After 5 minutes of stimulation, EGFR and Eps15 co-localized in a subset of EGFR-containing endosomal structures, mainly close to the plasma membrane (Fig. 7L).

To quantify the effect of expression of the truncated Eps15 mutants on recruitment of Eps15 to the plasma membrane, we scored at 1 minute after EGF stimulation 3×50 cells per condition for Eps15 membrane recruitment, as absent (–), intermediate (±) or strong (+) (Fig. 7M). The control cells and cells expressing UIM-mut showed either a strong or an intermediate recruitment of Eps15. In contrast, in the sample transfected with UIM-WT Eps15 was recruited to the plasma membrane in only a few cells, while most cells showed no Eps15 re-localization to the plasma membrane or an intermediate level.

In conclusion, the UIM-WT, which represses c-Cbl-induced EGFR endocytosis, inhibits EGF-induced recruitment of endogenous Eps15 towards the plasma membrane and into endosomes containing the activated EGFR. This inhibition depends on an intact second UIM, suggesting that UIM-WT competes with endogenous Eps15 in the binding of a ubiquitin moiety on a c-Cbl substrate. Thus, interaction between ubiquitin in this c-Cbl substrate and the UIM in Eps15 directs Eps15 into the endocytic route used by the activated EGFR.

Discussion

We found previously that c-Cbl binds to and ubiquitinates the EGFR at the plasma membrane, before its entry into clathrin coated vesicles (de Melker et al., 2001). This suggested that c-Cbl might play a role in the recruitment of the activated EGFR into CCP. Our present study further clarifies how c-Cbl facilitates the early steps of EGFR endocytosis. The EGFR appears to rely on the ubiquitin ligase function of c-Cbl to recruit Eps15 to the plasma membrane and to internalize via a clathrin-dependent pathway that involves Eps15. Based on our findings, we propose the following scenario: upon ligand binding, the EGFR kinase becomes activated and

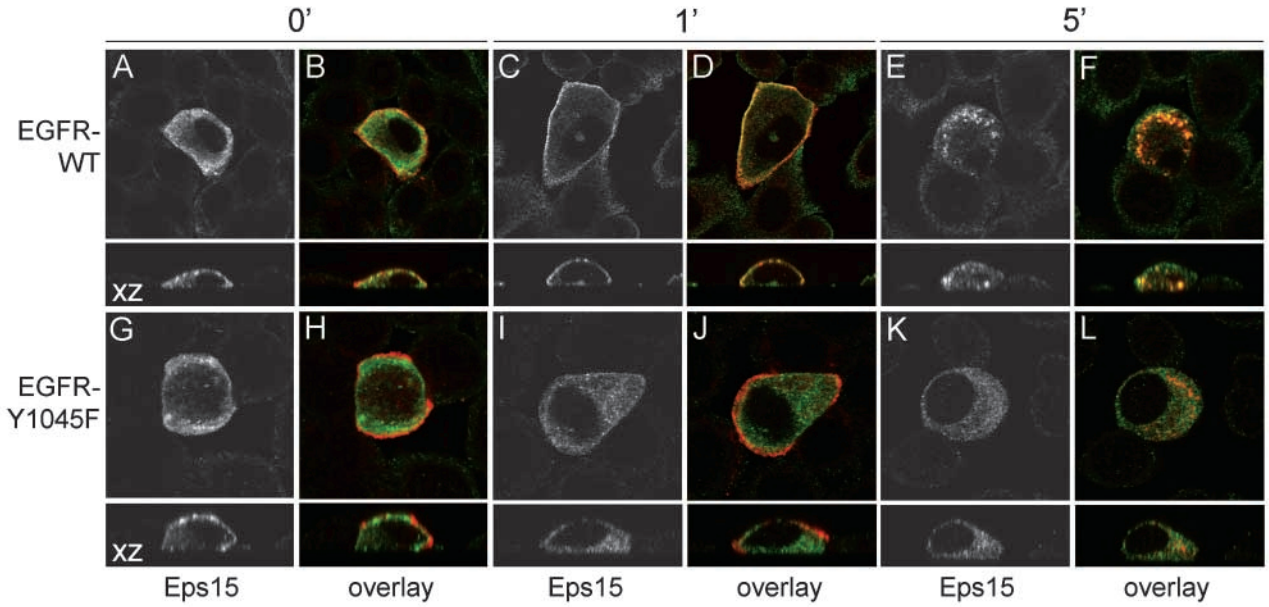

Fig. 4. EGF-induced re-distribution of Eps15 depends on Y1045 of the EGFR. CHO cells co-expressing FLAG-tagged Eps15, c-Cbl and wild-type EGFR (A-F) or EGFR-Y1045F (G-L) were incubated with EGF-TxR (red) on ice for 1 hour. After stimulation at 37°C for 0 minutes (A,B,G,H), 1 minute (C,D,I,J) or 5 minutes (E,F,K,L), cells were fixed and stained with anti-FLAG, followed by FITC-conjugated secondary antibody (green). Cells were analyzed by confocal microscopy. The displayed confocal planes are from the basal half of the cells. XZ sections are shown below each panel.

phosphorylates various tyrosine residues in the cytoplasmic tail of the receptor, including the c-Cbl binding sites (Y1045) and the Grb-2 binding site (Waterman et al., 2002). c-Cbl associates with Y1045 via its N-terminal PY-binding domain or alternatively with the Grb-2 binding site via its C-terminus, using Grb-2 as an intermediate. A ubiquitin conjugating enzyme (Ubc), associated with the Ring finger domain of c-Cbl, transfers mono-ubiquitin to the EGFR and to other substrates that are found in the activated EGFR complex. Ubiquitin links the activated EGFR complex to the UIM in Eps15. Eps15 either interacts directly with a ubiquitin moiety on the EGFR, or with another EGFR-associated substrate of the c-Cbl ubiquitin ligase. We postulate that, by virtue of its association with AP-2 (Benmerah et al., 1995), Eps15 recruits clathrin and a number of other structural and regulatory components involved in CCP formation (Marsh and McMahon, 1999). In this way, the ubiquitin ligase function of c-Cbl is instrumental in de novo formation of CCP that include activated EGFR.

Only recently, it was discovered that mammalian Eps15 and Eps15R, which bind the clathrin adaptor AP-2, contain a UIM (Polo et al., 2002). We are the first to show the participation of the UIM on an endocytic protein in ligand-induced receptor internalization in mammalian cells. In yeast, the involvement of UIM⁻containing proteins in receptor internalization has been demonstrated by deletion of the UIMs of the epsins Ent1p and Ent2p, which disrupted internalization of the α-factor (Shih et al., 2002). In addition, sorting of proteins from the

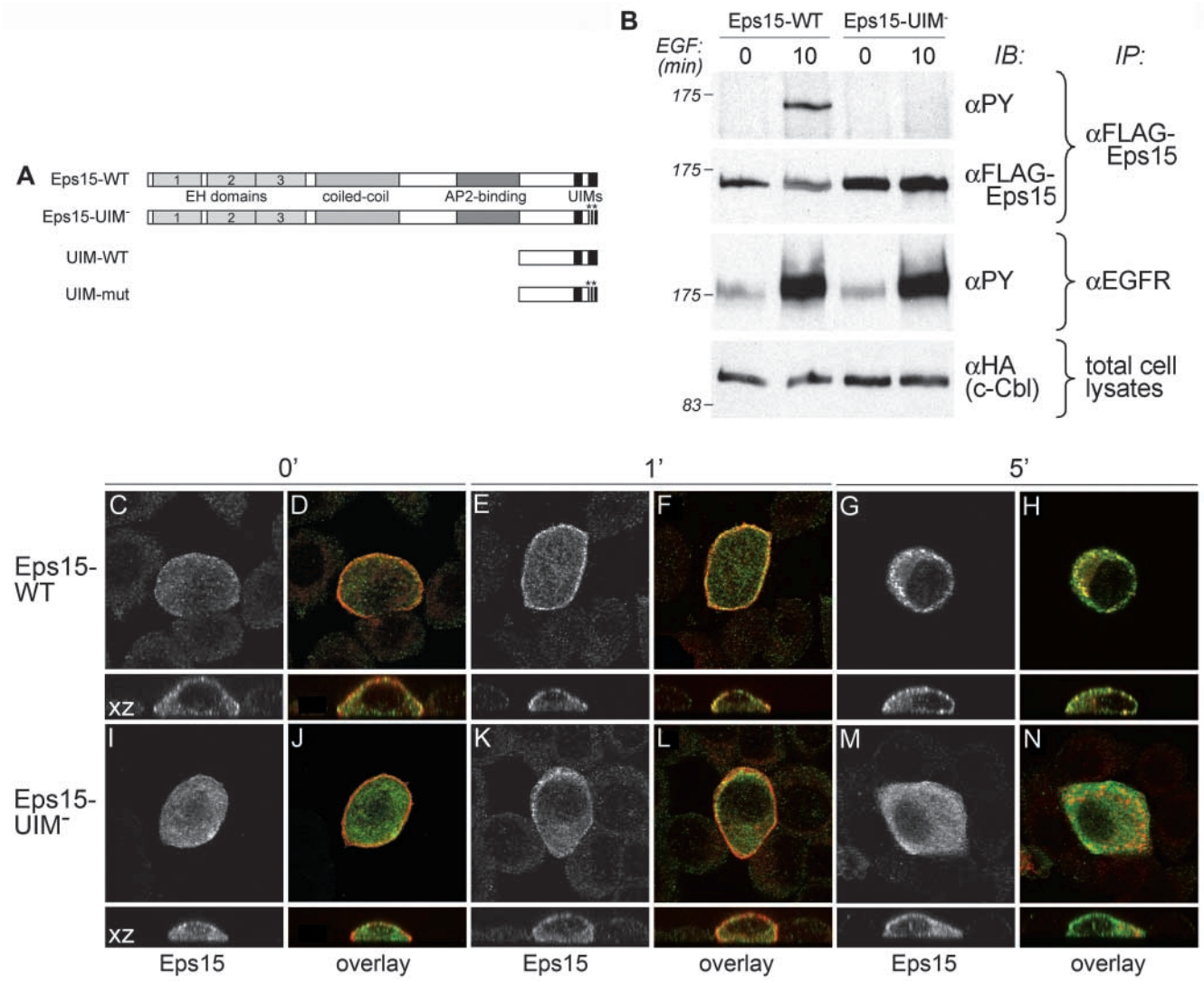

Fig. 5. Mutation of the second UIM of Eps15 abrogates EGF-induced Eps15 phosphorylation and re-distribution. (A) Wild-type and mutant Eps15 constructs used in this study. Eps15-UIM⁻ contains leucine to alanine substitutions at positions 883 and 885, indicated by an asterisk. UIM⁻WT consists of amino acids 741-897 of murine Eps15. UIM⁻mut in addition contains L883A/L885A substitutions. (B) CHO cells were co-transfected with EGFR-WT, c-Cbl and Eps15-WT or Eps15-UIM⁻. FLAG-tagged Eps15 and EGFR were immunoprecipitated from stimulated or control cells. Immunoprecipitates (IP) and total cell lysates were separated on gel and immunoblotted (IB) using mAbs to PY, FLAG-tag or HA-tag. (C-N) For immunofluorescence, CHO cells expressing EGFR-WT, c-Cbl and Eps15-WT (C-H) or Eps15-UIM⁻ (I-N) were incubated with EGF-TxR (red) on ice for 1 hour. After stimulation at 37°C for 0 minutes (C,D,I,J), 1 minute (E,F,K,L) or 5 minutes (G,H,M,N), cells were stained with anti-FLAG mAb, followed by FITC-conjugated secondary antibody (green). The displayed confocal planes are from the basal half of the cells. XZ sections are shown below each panel.

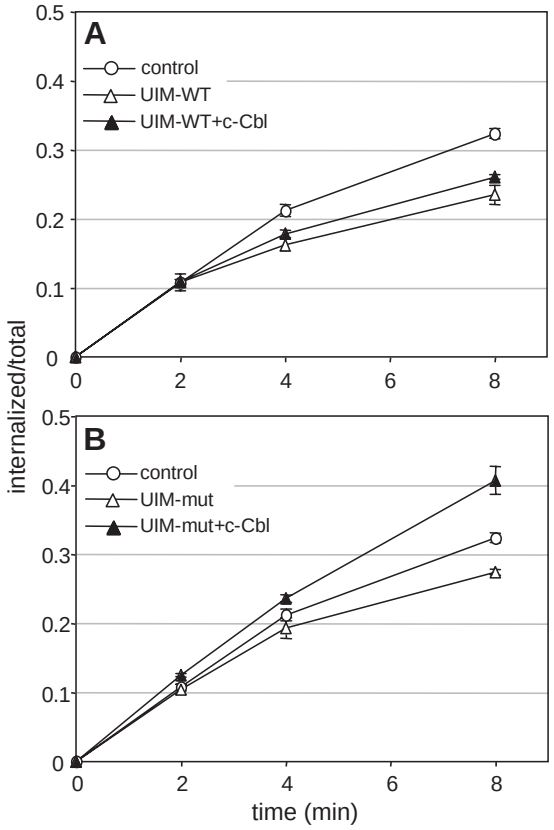

Fig. 6. Dominant negative effect of UIM⁻WT on c-Cbl-mediated EGFR internalization depends on intact UIM. CHO cells expressing the EGFR alone (○) or co-expressing the EGFR and FLAG-tagged UIM-WT (A) or UIM-mut (B) constructs (see Fig. 5A) in the presence (▲) or absence of c-Cbl (△) were incubated at 37°C with 1 ng/ml ^{125}I-labelled EGF. Internalization was determined as outlined in Materials and Methods. Presented is the ratio of internalized to total-cell-associated radioactivity. The experiments contained triplicate samples. Error bars represent standard deviations.

trans-Golgi network, as well as into the inner vesicles of multivesicular endosomes, was found to depend on protein ubiquitination and ubiquitin receptors. For example, the UIM of Vps27p, the yeast homologue of Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate), is required for the sorting of ubiquitinated proteins, both biosynthetic and endocytic, from the limiting membrane into internal vesicles of multi-vesicular bodies (Shih et al., 2002). In mammalian cells, Hrs sorts transferrin receptors fused to ubiquitin, but not wild-type receptors, from endosomes into the degradative pathway (Raiborg et al., 2002). Although we have focused here on a role of c-Cbl at the plasma membrane, c-Cbl remains associated with the activated EGFR throughout the endocytic route (de Melker et al., 2001). It is well established that c-Cbl also plays a role post-endocytosis. It enhances receptor sorting towards lysosomes, which appears to depend on its ubiquitination function (Levkowitz et al., 1998; Longva et al., 2002). Thus, there appear to be many parallels between the mechanism of receptor internalization at the plasma membrane and protein sorting in endosomes.

Upon discovery of the UIM in Eps15(R) (Polo et al., 2002), Riezman (Riezman, 2002) proposed a model, in which mammalian Eps15(R) would form a bridge between the ubiquitinated EGFR and the clathrin lattice and thus promote entry of activated EGFR into CCP. We here provide experimental support for this model. However, an important 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 (Torrisi et al., 1999; van Delft et al., 1997), we and others (Fazioli et al., 1993; Sorkina et al., 1999; Torrisi et al., 1999) have not been able to show this. Generally, only a low proportion of activated EGFR is ubiquitinated, suggesting that ubiquitination is transient. This would also result in a transient interaction between Eps15 and the EGFR, which may be difficult to visualize. Using an EGFR-ubiquitin chimera, Dikic and colleagues showed that a single ubiquitin is sufficient to allow for ligand-independent receptor internalization (Haglund et al., 2003b). However, in case of the growth hormone receptor, recruitment of the ubiquitination machinery, but not ubiquitination of the receptor itself, is essential for internalization (Govers et al., 1999), suggesting a role for other ubiquitinated proteins as docking site for the endocytic machinery. Because the ubiquitination sites in the EGFR are undefined, we cannot use ubiquitination-deficient EGFR mutants to assess whether ubiquitin moieties on the EGFR itself are the key docking sites for Eps15; so it is equally possible that another substrate of the c-Cbl ubiquitin ligase is involved.

There is evidence that c-Cbl can promote internalization of the EGFR in the absence of ubiquitination of the EGFR itself. EGFR ubiquitination is mainly dependent on the presence of Y1045 in the EGFR tail (Levkowitz et al., 1999; Waterman et al., 2002). Apparently, only binding of c-Cbl to phosphorylated Y1045 positions it appropriately to target the unknown ubiquitination sites in the EGFR. Nevertheless, c-Cbl can promote internalization of an EGFR mutant that lacks Y1045, presumably in the absence of receptor ubiquitination. Although the effect of the Y1045F mutation on EGFR internalization in some cell types is small (Jiang and Sorkin, 2003), we and others find that it consistently reduces EGFR internalization (Waterman et al., 2002). Furthermore, we present here that mutation of Y1045 results in an inhibition of EGF-induced Eps15 phosphorylation, and co-localization of EGFR and Eps15. However, it had consistently less impact on these processes than over-expression of 70Z-Cbl, which blocks binding to Y1045 as well as to the Grb-2 site. It has been found that an EGFR Y1045 mutant and c-Cbl can still co-localize in CCP (Jiang and Sorkin, 2003). In the porcine aortic endothelial cells these authors used, c-Cbl appeared to regulate EGFR internalization primarily via the Grb-2 binding site. In this case, endocytosis still depended on the Ring finger of c-Cbl. This is consistent with the idea that the ubiquitin ligase function of c-Cbl can promote EGFR internalization in the absence of EGFR ubiquitination.

It should be emphasized that the N-terminal PY-binding domain is the conserved feature in all Cbl proteins, including mammalian c-Cbl, Cbl-b and Cbl-3, as well as Cbl in *Drosophila* (D-Cbl) and *C. elegans* (Sli-1) (Tsygankov et al., 2001; Smit and Borst, 1997). Cbl-3 and Sli-1 lack the proline stretch involved in Grb-2 binding. Therefore, we suggest that during evolution, some Cbl proteins have acquired the

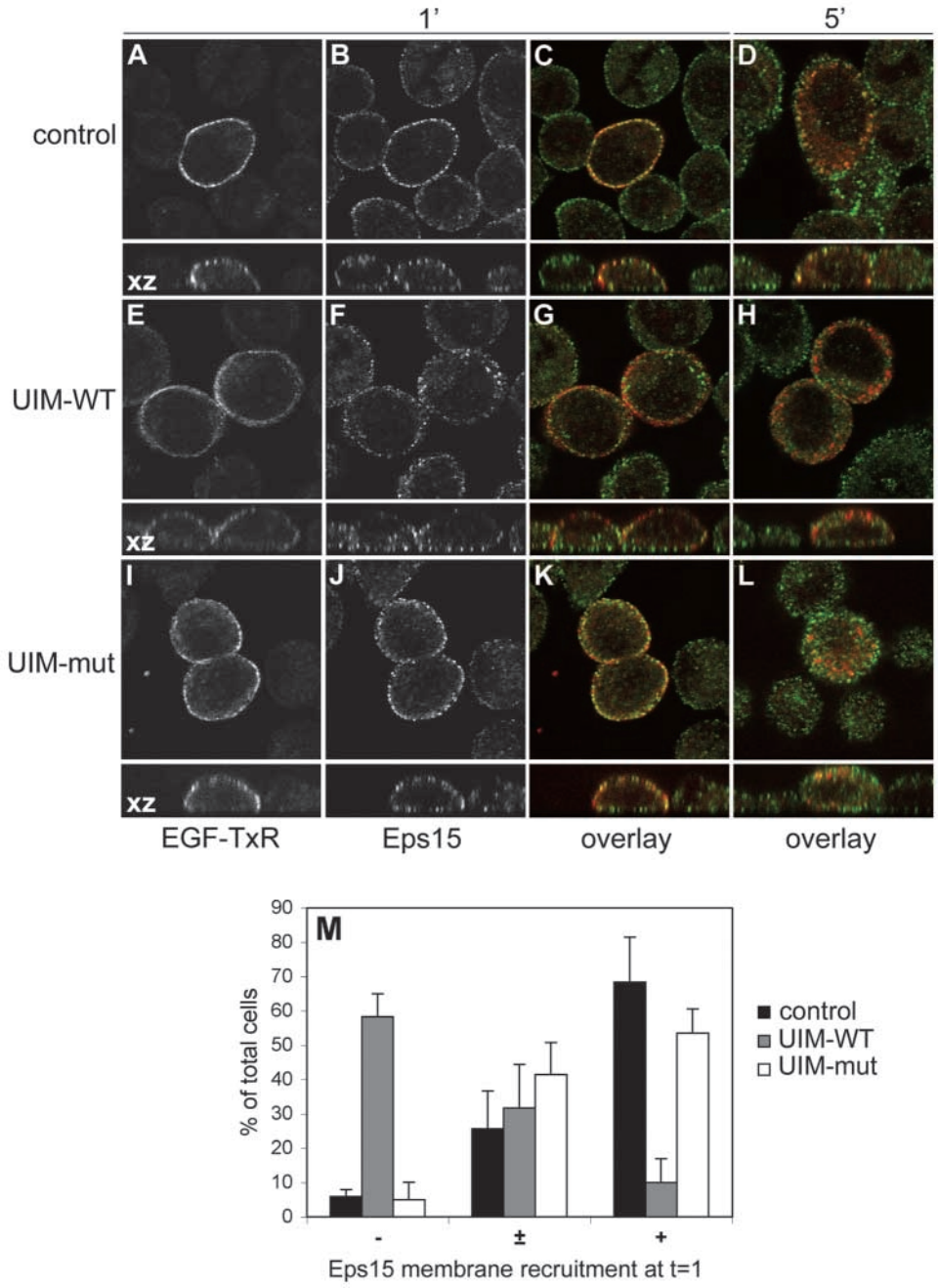

Fig. 7. UIM-WT, but not UIM-mut, inhibits EGF-induced re-distribution of endogenous Eps15. CHO cells expressing EGFR-WT together with an empty vector (A-D), FLAG-UIM-WT (E-H) or FLAG-UIM-mut (I-L) were incubated with EGF-TxR (red) on ice for 1 hour. After stimulation at 37°C for 1 minute (A-C, E-G, I-K) or 5 minutes (D,H,L), cells were stained with anti-Eps15 pAb, followed by FITC-conjugated secondary antibodies (green). Cells were analyzed by confocal microscopy. The displayed confocal planes are from the basal half of the cells, except for panel (L), which is from the upper part of the cell. XZ sections are shown below each panel. (M) For each transfectant used in the confocal experiment described above, 3×50 cells were analyzed at t=1 minute and scored for plasma membrane recruitment of Eps15. Black bars, control cells; grey bars, UIM-WT-expressing cells; white bars, UIM-mut-expressing cells. Error bars represent standard deviations between the three independent analyses.

additional capacity to facilitate endocytosis via the Grb-2 binding mode and the ubiquitination of a substrate other than the EGFR itself. An interesting candidate is CIN85, which is constitutively bound to the distal C-terminus of c-Cbl and mono-ubiquitinated by c-Cbl (Haglund et al., 2002). CIN85 recruits endophilin to the plasma membrane and in this way may also facilitate receptor internalization by promoting membrane curvature (Soubeyran et al., 2002).

Mutation of the UIM of Eps15 has a dual effect: it disrupts binding to ubiquitinated proteins as well as Eps15 mono-ubiquitination (Polo et al., 2002). These two effects are not separable. Therefore, we cannot exclude that the effect of the UIM mutation on Eps15 phosphorylation and recruitment is due to absence of Eps15 ubiquitination. Although less likely, the dominant negative effect of the Eps15 UIM may also depend on ubiquitination rather than binding to ubiquitinated proteins. Eps15 is not ubiquitinated by c-Cbl, but by Nedd4, a ubiquitin ligase of the HECT subfamily (Polo et al., 2002). If mono-ubiquitination of Eps15 is involved in c-Cbl-enhanced receptor internalization, this would implicate the involvement of additional proteins that contain ubiquitin-binding domains and would complicate the model considerably. Nevertheless, it would not change the idea that the capacity of c-Cbl to direct receptor internalization is based on ubiquitin-UIM interactions.

Effects of c-Cbl on receptor sorting after the formation of endocytic vesicles are well established (Levkowitz et al., 1998). Distorting effects of receptor recycling in the

internalization experiments are unlikely, because we found significant impact of c-Cbl and its mutants from the 4-minute time point onwards. Using Eps15 tyrosine phosphorylation and recruitment of Eps15 to the plasma membrane as additional read outs, we have established that the ubiquitination function of c-Cbl is relevant for EGFR endocytosis at very early time points (1-2 minutes), when recycling does not yet come into play.

It has been argued that EGF concentrations are critical in the type of internalization experiments performed in this study, because high concentrations of EGF may lead to saturation of the clathrin-dependent route and may favour other modes of internalization. We have performed our internalization experiments at high (20 ng/ml, not shown) and low (1 ng/ml) concentrations of EGF and found comparable effects of c-Cbl and 70Z-Cbl on EGFR internalization in CHO cells. Moreover, at 20 ng/ml EGF, all EGFR internalization observed in CHO cells was still clathrin-dependent as determined by K^+ depletion (Fig. 1F). Clearly, a large component of clathrin-dependent EGFR internalization in these cells is independent of Cbl molecules, because the EGFR was still endocytosed in the presence of 70Z-Cbl and entered efficiently into endosomes. It should be emphasized, therefore, that our present study does not implicate c-Cbl as an essential requirement for clathrin-mediated endocytosis of the EGFR.

The extent to which Cbl contributes to EGFR internalization is clearly cell-type dependent. For instance, 70Z-Cbl efficiently downregulated EGFR internalization in Hela and NIH3T3 cells, but to a lesser extent in porcine aortic endothelial cells (Jiang and Sorkin, 2003; Thien et al., 2001). This argument may also explain why in c-$Cbl^{-/-}$ mouse embryonic fibroblasts EGFR internalization was not significantly reduced and why ubiquitination-deficient Chinese hamster lung cells could still internalize EGFR (Duan et al., 2003). The significance of our study lies in characterising the mechanism of action of c-Cbl. Namely, it is the ubiquitin ligase activity of c-Cbl that promotes EGFR endocytosis. It does so by shuttling the EGFR into a clathrin-mediated endocytic pathway that involves Eps15.

In absence of the ubiquitination function of c-Cbl, the EGFR does internalize via a clathrin-dependent route, but we do not observe any Eps15 in this route. Although a multitude of (sub)membrane structures containing endogenous Eps15 were present, the EGFR did not make use of them in absence of c-Cbl-mediated UIM interactions. This suggests that, in absence of the c-Cbl ubiquitin ligase, the EGFR internalizes via a clathrin-mediated (but Eps15-independent) route. Presumably, this pathway also does not make use of Eps15R, which is expected to be recruited to the EGFR in a ubiquitin-dependent manner as well. Experiments with Eps15 mutants suggest that Eps15 is a requirement for EGFR endocytosis. Over-expression of Eps15 mutants that contain an intact AP-2 binding domain (but cannot participate in CCP) strongly impaired ligand-induced EGFR endocytosis (Benmerah et al., 1999; Benmerah et al., 1998; Benmerah et al., 2000). It should be realized however, that over-expression of cytosolic Eps15 may interfere with Eps15-independent endocytic pathways as well. It may do so by sequestering AP-2 and/or other proteins important for CCP formation. Selective knockdown of Eps15(R) expression by RNA interference is therefore a more appropriate way to find out whether the EGFR receptor can be internalized via an Eps15-independent pathway.

Although we have concentrated in this study on c-Cbl and the EGFR, we expect that internalization of all tyrosine-kinase-based receptor systems can be facilitated by members of the Cbl family through the establishment of critical ubiquitin-UIM interactions. This may involve not only Eps15(R), but also other ubiquitin receptors and ubiquitin ligase substrates that are instrumental in clathrin-mediated receptor endocytosis.

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