c-Cbl ubiquitinates the EGF receptor at the plasma membrane and remains receptor associated throughout the endocytic route

Annemieke A. de Melker[1], Gerda van der Horst[1], Jero Calafat[2], Hans Jansen[2] and Jannie Borst[1,*]

[1]Division of Cellular Biochemistry, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands
[2]Division of Cell Biology, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands
*Author for correspondence (e-mail: jborst@nki.nl)

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SUMMARY

Cbl family members have an evolutionarily conserved role in attenuating receptor tyrosine kinase function. Their negative regulatory capacity depends on a Ring finger domain that interacts with ubiquitin conjugating enzymes. Cbl molecules constitute a novel type of E3 or ubiquitin ligase family that is recruited to phosphotyrosine motifs. Ubiquitination of the receptor system is coupled to its downregulation, but it is unclear at which point in the endocytic pathway Cbl molecules come into play. Using low temperature and a dynamin mutant, we find that c-Cbl associates with and ubiquitinates the activated epidermal growth factor (EGF) receptor at the plasma membrane in the absence of endocytosis. With the aid of confocal microscopy and immunogold electron microscopy, we could demonstrate that c-Cbl associates with the EGF receptor at the plasma membrane prior to receptor recruitment into clathrin-coated pits and remains associated throughout the clathrin-mediated endocytic pathway. c-Cbl and the EGF receptor also colocalize in internal vesicles of multivesicular endosomes. Our data are consistent with a role for c-Cbl in clathrin-mediated endocytosis of tyrosine kinase receptors, as well as their intracellular sorting.

Key words: EGF receptor, Endocytosis, Cbl, Downregulation, Ubiquitin

INTRODUCTION

Ligand binding to a diverse group of membrane receptors, including receptor tyrosine kinases (RTKs), cytokine receptors and heterotrimeric guanine nucleotide binding protein-coupled receptors, enhances endocytosis of the ligand-receptor complex. Endocytosis promotes removal of receptors from the cell surface in order to (1) relocate activated receptors to specific intracellular sites from which signalling is propagated and (2) attenuate signal transduction (Ceresa and Schmid, 2000; Sorkin, 1998). Mechanisms of receptor downregulation have been best studied for RTKs, and especially the epidermal growth factor receptor (EGFR). In the absence of ligand, a large fraction (40-60%) of EGFRs is found in caveolae, which are membrane microdomains that have a specialized composition of lipids and proteins (Carpenter, 2000). Increased endocytosis of EGFRs results from relocation of the ligand-receptor complex to clathrin-coated pits, the starting points of clathrin-mediated internalization. From there, receptors are sorted to the lysosomal degradation pathway (Carpenter, 2000; Sorkin, 1998). In the absence of ligand, EGFRs are constitutively endocytosed and recycled back to the cell surface, but not specifically recruited to clathrin-coated pits. Defective downregulation of growth stimulatory receptors might result in malignant transformation of cells, emphasizing the importance of the molecules regulating this process (Di Fiore and Gill, 1999).

The cellular homologue of the murine retroviral protein v-Cbl, c-Cbl (Langdon et al., 1989), is a negative regulator of a wide variety of protein tyrosine kinase-coupled membrane receptors. Three mammalian *Cbl* genes have been identified (Blake et al., 1991; Keane et al., 1995; Keane et al., 1999; Kim et al., 1999) and Cbl homologues have been found in *Caenorhabditis elegans* (Yoon et al., 1995) and *Drosophila* (Meisner et al., 1997; Robertson et al., 2000). The first evidence that Cbl molecules negatively regulate RTKs came from studies in *C. elegans*, in which loss of function of the Cbl homologue Sli-1 compensated for defective Let-23 (EGFR) signalling (Jongeward et al., 1995). *Drosophila* Cbl (D-Cbl) was found to suppress the development of the R7 photoreceptor neuron, which is guided by RTK signalling (Meisner et al., 1997). c-Cbl deficient mice show several morphological aberrations that indicate loss of cell growth control, such as hyperplasia of lymphoid and mammary tissues and abundant extramedullary haematopoiesis in the spleen (Murphy et al., 1998). In addition, c-Cbl$^{-/-}$ thymocytes express higher T cell antigen receptor levels and show enhanced ZAP-70 activity compared to wild-type cells, suggesting a defect in negative control of receptor signalling (Thien et al., 1999). In Cbl-b$^{-/-}$ mice, the signalling threshold for antigen-specific T cell activation is lowered and reached in the absence of CD28 signals (Bachmaier et al., 2000; Chiang et al., 2000). An explanation for the negative regulatory function of Cbl molecules was provided by the observation that c-Cbl

stimulates downregulation of receptor numbers at the plasma membrane (Lee et al., 1999; Levkowitz et al., 1998; Miyake et al., 1998).

The N-terminal half of the Cbl proteins is conserved during evolution and contains a phosphotyrosine-binding region and a Ring finger domain. The phosphotyrosine-binding region is composed of a four-helix domain, an EF hand and an SH2-like domain (Meng et al., 1999), and is recruited to phosphotyrosine motifs in RTKs and the ZAP-70/Syk-family of cytoplasmic tyrosine kinases (Smit and Borst, 1997). Recently, it has been found that the Ring finger of c-Cbl interacts with ubiquitin-conjugating proteins, or E2s, and that c-Cbl is in fact a novel type of E3, or ubiquitin ligase, that is recruited to and ubiquitinates tyrosine-kinase-coupled membrane receptor systems (Joazeiro et al., 1999; Levkowitz et al., 1999; Yokouchi et al., 1999). Overexpression of c-Cbl strongly enhances ubiquitination and degradation of the EGFR, platelet-derived growth factor receptor and colony stimulating factor 1 receptor (CSF-1R) (Lee et al., 1999; Levkowitz et al., 1998; Miyake et al., 1998). c-Cbl mutants lacking a functional Ring finger domain cannot mediate receptor ubiquitination and downregulation. Such mutants include the oncogenic variants v-Cbl as well as 70Z-Cbl, which was originally isolated from the 70Z/3 mouse pre-B-lymphoma cell line and contains a 17 amino acid deletion that disrupts the Ring finger motif (Andoniou et al., 1994).

It is not clear at which point in the endocytic pathway c-Cbl comes into play, nor how it promotes the removal of receptors from the cell surface. It has been proposed that it interferes with recycling by stimulating receptor sorting to the lysosomes. In that study, c-Cbl was found to associate with the EGFR upon its arrival in endosomes (Levkowitz et al., 1998). Other investigators suggest that c-Cbl stimulates endocytosis per se. Upon CSF-1 stimulation of macrophages, c-Cbl was targeted to the plasma membrane (Wang et al., 1996) and studies on macrophages of c-Cbl$^{-/-}$ mice indicated that c-Cbl promotes rapid internalization of the CSF-1R (Lee et al., 1999).

We have analysed the spatiotemporal interaction between c-Cbl and the EGFR after ligand binding. We show that c-Cbl is recruited to and ubiquitinates the activated EGFR while it is still present at the cell surface. Using confocal and electron microscopy, we find c-Cbl together with the EGFR at the plasma membrane, as well as in clathrin-coated vesicles and in multivesicular bodies. Our data indicate that c-Cbl might be involved in the recruitment of the EGFR into clathrin-coated pits, as well as in sorting of the receptor to the lysosomes.

MATERIALS AND METHODS

Cell culture and antibodies

COS-7 and Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% foetal calf serum (FCS), penicillin and streptomycin. Antibodies used were: affinity purified rabbit polyclonal IgG detecting a C-terminal epitope in c-Cbl (C-15, Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit polyclonal serum 282.7 directed against the EGFR (L. H. Defize, Hubrecht Laboratory, Utrecht, The Netherlands); rabbit polyclonal anti-peptide serum RK-2 recognizing the EGFR C-terminal region (Kris et al., 1985) (J. Schlessinger, NYU Medical Center, New York, NY, USA); the mouse monoclonal antibodies (mAb) 528 (Gill et al., 1984) (American Type Culture Collection) and 108.1 (Bellot et al., 1990), both directed against the EGFR; rabbit anti-clathrin-heavy-chain polyclonal antiserum (S. Corvera, University of Massachusetts, Worcester, MA, USA); mAb 23 directed against the clathrin heavy chain (Transduction Laboratories, Lexington, KY, USA); mAb 41 directed against dynamin (Transduction Laboratories) and mAb 12CA5 against the hemagglutinin (HA)-tag (Wilson et al., 1984). For enhanced chemiluminescence, horseradish-peroxidase-conjugated rabbit anti-mouse or swine anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark) were used. For immunofluorescence, FITC-conjugated goat anti-mouse or goat anti-rabbit IgG (Rockland, Gilbertsville, PA, USA) and Texas-Red-conjugated goat anti-mouse (Rockland, Gilbertsville, PA, USA) or goat anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands) were used.

Constructs

The pMT2-HA-cCbl vector was generated by ligation of the HA-tagged *c-cbl* cDNA from pGEM4Z-cCbl (provided by W. Y. Langdon, University of Western Australia, Nedlands, Australia) into pMT2SM using its *Sal*I site. The pMT123 vector containing HA-*ubiquitin* cDNA has been described by Treier et al. (1994). The insert was isolated using *Eco*RI/*Not*I and ligated into pMT2SM. The pMT2-dynamin-1 (wild type and K44A mutant) constructs have been described elsewhere (Kranenburg et al., 1999). The pMT2-EGFR vector was a gift of W. Moolenaar (The Netherlands Cancer Institute, Amsterdam, The Netherlands).

Transfections

COS cells (1×10^6 per 10 cm dish) were transfected by the DEAE-dextran method, using routinely 2.5 μg DNA per plasmid. CHO cells (3×10^5 per 3.5 cm well) were transfected using lipofectamine PLUS Reagent (Gibco). The following quantities of DNA were used for each construct: 3 μg of pMT2-HA-cCbl, 2 μg of pMT2-EGFR, 1 μg of pMT2-HA-ubiquitin and 4 μg of the dynamin cDNAs. The DNA mixture was added to the cells in a total volume of 1 ml of DMEM. After 4-6 hours, the DNA containing solution was removed and 3 ml medium were added. CHO cells stably expressing the human EGFR were generated by transfecting 1 μg pMT2-EGFR with 1 μg of pcDNA3 to allow selection on medium containing 2.5 mg ml^{-1} G418. Cells strongly expressing the EGFR were selected after 3 weeks by sorting with a MoFlo high speed sorter (Cytomation, Fort Collins, CO, USA) using anti-EGFR mAb 108.1.

Immunoprecipitation and immunoblotting

Cells were used for immunoprecipitation 48 hours after transfection. COS cells were serum starved overnight and stimulated with 25 ng ml^{-1} EGF (Becton Dickinson Labware, Bedford, MA, USA) on ice or at 37°C for several periods of time. After stimulation, cells were immediately put on ice, washed quickly with cold PBS and incubated for 30 minutes at 0°C with lysis buffer (1% NP-40, 30 mM Tris-HCl pH 7.5, 150 mM NaCl, 4 mM EDTA pH 8.0, 10 mM NaF, 1 mM sodium orthovanadate, 10 μg ml^{-1} phenylmethylsulfonyl fluoride, 0.1 μM leupeptin and 0.1 μM aprotinin). Cell lysates were clarified by centrifugation for 10 minutes at 14,000 rpm. The appropriate antibodies were added to the lysates and incubated for 2 hours or overnight at 4°C. Immune complexes were incubated with protein A-Sepharose beads for an additional 2 hours. Precipitated proteins were subjected to SDS-PAGE and blotted to nitrocellulose. Filters were blocked for 30 minutes at room temperature or at 4°C overnight in TBST (20 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.05% Tween-20) containing 1% bovine serum albumin (BSA) or non-fat dry milk. Filters were incubated with primary antibodies for 2 hours at room temperature or overnight at 4°C, washed three times in TBST and incubated with secondary antibodies (1:7500 dilution of horseradish-peroxidase-conjugated rabbit anti-mouse Ig or swine anti-rabbit Ig) for 45 minutes at room temperature. After washing the filters in

TBST, proteins were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Immunofluorescence

One day after transfection, cells were detached with trypsin/EDTA and plated on coverslips placed in six-well plates. Cells were allowed to attach for 24 hours and then serum starved for 3 hours (CHO) or overnight (COS) in DMEM containing 20 mM HEPES and 0.1% BSA. Cells were stimulated with 25 ng ml^{-1} EGF at 37°C or on ice for the appropriate periods of time. When stimulation was performed on ice, cells were incubated for 30 minutes on ice prior to the addition of EGF. For cytosol depletion, stimulated cells were treated for 15 minutes on ice with 0.05% saponin in permeabilization buffer (80 mM PIPES pH 6.8, 0.1 mM EGTA and 0.5 mM $MgCl_2$) prior to fixation. Cells were fixed on ice for 2 minutes with methanol kept at –20°C. Methanol was removed and coverslips were allowed to dry at room temperature. All further incubations were performed at room temperature. Cells were rehydrated in PBS for 5 minutes and nonspecific binding sites were blocked for 30 minutes using 1% BSA in PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Incubations were performed with antibodies diluted in blocking buffer for 45 minutes, after which coverslips were washed and incubated for 30 minutes with the appropriate FITC- or Texas-Red-conjugated secondary antibodies diluted in blocking buffer. Coverslips were washed and mounted in Vectashield (Vector Laboratories, Burlingham, CA, USA) and viewed under a Leica TCS NT confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany). Confocal images were taken from a basal plane of the cells, just above the basal membrane, unless indicated otherwise.

Immunoelectron microscopy

COS cells plated in 10 cm petri dishes were transfected with *c-cbl* cDNA as described above. Cells were serum starved overnight and stimulated with 25 ng ml^{-1} EGF at 37°C or on ice. Cells were fixed for 24 hours in 4% paraformaldehyde in 0.1 M PHEM buffer (80 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$ and 10 mM EGTA, pH 6.9) and processed for ultrathin cryosectioning as described (Calafat et al., 1997). 45-nm cryosections were cut at –125°C using diamond knives (Drukker Cuijk, The Netherlands) in an ultracryomicrotome (Leica Aktiengesellschaft, Vienna, Austria) and transferred with a mixture of sucrose and cellulose onto formvar-coated copper grids (Liou et al., 1996). The grids were placed on 35-mm petri dishes containing 2% gelatin. For single immunolabelling, the sections were incubated with antibodies for 45 minutes, followed by 30 minutes incubation with 10-nm protein-A-conjugated colloidal gold (Department of Cell Biology, Utrecht University Medical Center, Utrecht, The Netherlands). For double immunolabelling, the procedure described by Slot et al. (1991) was followed with 10-nm and 15-nm protein-A-conjugated colloidal gold probes. As control, primary antibody was replaced by irrelevant rabbit antiserum. After immunolabelling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM 10 electron microscope (Eindhoven, The Netherlands).

RESULTS

c-Cbl associates with and ubiquitinates the EGFR in the absence of endocytosis

Interaction between c-Cbl and the EGFR was studied in COS and CHO cells, in which EGFR trafficking from the plasma membrane into and through the endocytic route is well characterized. In these cells, the EGFR is internalized via clathrin-coated pits. To examine whether c-Cbl participates in this process, its localization relative to clathrin was examined by confocal microscopy. COS cells expressing endogenous EGFR were transiently transfected with human c-Cbl. Because overexpressed c-Cbl is predominantly located in the cytoplasm, COS cells were permeabilized with saponin prior to fixation. This allows free cytosolic proteins to diffuse out of the cells, while membrane-associated proteins are retained. In unstimulated cells, anti-clathrin antibodies showed a punctate staining at the cell surface and the trans-Golgi network. No colocalization with the EGFR or c-Cbl could be detected (Fig. 1A,C). Five minutes after EGF stimulation, both the EGFR and c-Cbl localized at clathrin-coated structures (Fig. 1B,D). These observations indicate that c-Cbl might participate in clathrin-mediated endocytosis of the EGFR and comes into play prior to vesicle uncoating.

To establish at which point in the endocytic process c-Cbl associates with the EGFR, the distribution of both proteins was followed in time, under normal conditions and under conditions in which receptor internalization was blocked. When incubation with EGF is performed at 4°C or on ice, the

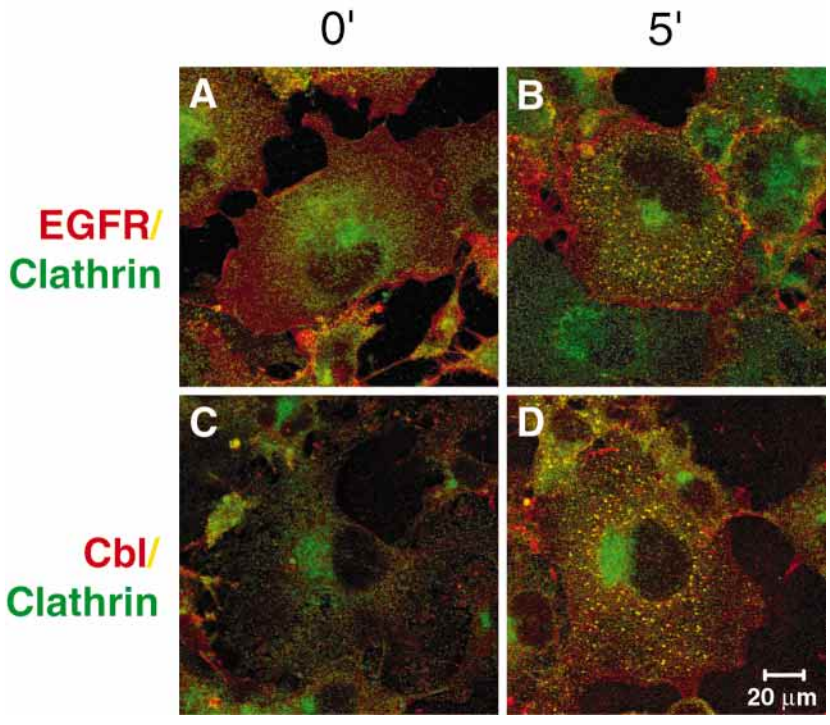

Fig. 1. Colocalization of the EGFR and c-Cbl with clathrin in stimulated cells. Serum starved COS-7 cells transiently expressing human c-Cbl were incubated without (A,C) or with (B,D) 25 ng ml^{-1} EGF for 5 minutes at 37°C. Prior to fixation, cells were permeabilized with saponin. Cells were incubated with antibody to the EGFR (polyclonal antibody RK-2) (A,B) or c-Cbl (C,D), followed by Texas-Red-conjugated secondary antibody and double stained with an antibody to clathrin (mAb 23) followed by FITC-conjugated anti-mouse Ig. Merged confocal images of the EGFR or c-Cbl (red) and clathrin (green) are shown.

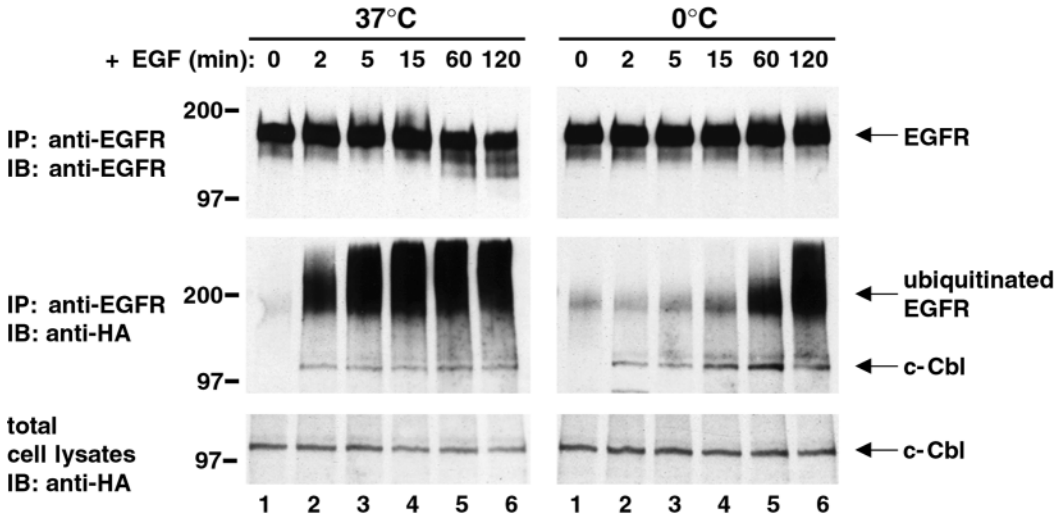

Fig. 2. c-Cbl associates with and ubiquitinates the EGFR at 0°C. HA-tagged ubiquitin and HA-tagged c-Cbl were transiently overexpressed in COS-7 cells. Serum starved cells were stimulated with 25 ng ml^{-1} EGF for the indicated time periods on ice or at 37°C. Immunoprecipitates (IP) of the endogenous EGFR (mAb 528) and total cell lysates were separated on 6% SDS-polyacrylamide gels. Immunoblotting (IB) was used to detect EGFR (polyclonal antibody 282.7), c-Cbl and ubiquitin (both anti-HA).

EGFR can still be activated (Galcheva-Gargova et al., 1995) but its internalization is prohibited (Beguinot et al., 1984; Haigler et al., 1979; Hanover et al., 1984; Hertel and Perkins, 1987). Therefore, we determined whether c-Cbl can be recruited to the EGFR and ubiquitinate it at such a low temperature. COS cells transiently expressing c-Cbl and HA-tagged ubiquitin were stimulated with EGF at 37°C or on ice. Anti-HA immunoblotting of anti-EGFR immunoprecipitates showed that, at 37°C, the EGFR associates with c-Cbl and is ubiquitinated within 2 minutes of EGF stimulation (Fig. 2). When stimulation was performed on ice, association between the EGFR and c-Cbl could also be detected within 2 minutes of EGF stimulation (Fig. 2). An increasing amount of c-Cbl was coprecipitated with the receptor in time, which might be explained by accumulation of ligand-receptor complexes at the cell surface. Ligand-induced association of c-Cbl with the EGFR was also detected by anti-Cbl immunoblotting of anti-EGFR immunoprecipitates (not shown). Ubiquitination of the EGFR clearly took place, be it with slower kinetics than at 37°C. It became detectable 15 minutes after EGF addition and increased during the 120-minute time frame of stimulation. Both at 37°C and on ice, ubiquitination was reflected by the appearance of a high molecular weight smear, as detected by immunoblotting with anti-EGFR antibody (Fig. 2). EGFR degradation products were, however, only detected after EGF stimulation at 37°C (60 and 120 minute time points), and not after stimulation on ice (Fig. 2). This indicates that the EGFR was not transported to intracellular degradation compartments at 0°C.

Because the kinetics of EGFR ubiquitination were delayed at 0°C, we used an independent method to block receptor internalization to see whether ubiquitination and endocytosis were coupled. In cells expressing a GTPase defective mutant of dynamin, K44A, coated pits do not become constricted and coated vesicles do not bud off into the cytoplasm (Damke et al., 1994; van der Bliek et al., 1993). Overexpression of this K44A mutant did not affect the efficiency or kinetics of the association of c-Cbl with the activated EGFR (Fig. 3A). Also, ubiquitination of the EGFR was not impeded in cells overexpressing dynamin-K44A. Rather, ubiquitination was more prominent 15 minutes after EGF addition than in the absence of the dynamin mutant. Overexpression of wild-type dynamin did not affect association of c-Cbl with the EGFR or its ubiquitination. Ligand induction of the association between c-Cbl and the EGFR was verified using antibodies against c-Cbl for immunoblotting (not shown). Confocal microscopy demonstrated the effectiveness of the K44A mutant (Fig. 3B). In cells transfected with wild-type dynamin cDNA, the EGFR was present in endosomes 5 minutes after EGF addition, whereas receptor internalization was blocked in cells expressing the K44A mutant. From the collected experiments, we conclude that c-Cbl associates with and ubiquitinates the activated EGFR prior to its internalization from the plasma membrane.

c-Cbl and the EGFR colocalize throughout the transport route from plasma membrane to late endosomes

To define the stages in the endocytic route of the EGFR at which c-Cbl might play a role, we studied the localization of c-Cbl and the activated EGFR by confocal microscopy. COS cells were transfected with HA-tagged *c-cbl* cDNA, plated on coverslips, stimulated with EGF at 37°C or on ice and treated with saponin prior to fixation. As noted above, this method leads to the selective loss of cytoplasmic c-Cbl. In unstimulated cells, c-Cbl staining was therefore very weak, whereas the EGFR was found on the plasma membrane with a strong presence in lamellipodia (Fig. 4A). Stimulation with EGF for 2 minutes at 37°C induced membrane ruffles (Fig. 4D-F) (Bretscher, 1989; Bretscher and Aguado-Velasco, 1998; Connolly et al., 1984), and very clear translocation of c-Cbl to the plasma membrane (Fig. 4E). The EGFR and c-Cbl colocalized in the membrane ruffles (Fig. 4F). Longer incubations with EGF (5-30 minutes) at 37°C resulted in internalization of the EGFR. Colocalization of c-Cbl and the EGFR in endosomes has been described by others (Levkowitz et al., 1998; Lill et al., 2000) and is not shown here. After 60 minutes of cell stimulation at 37°C, the EGFR was present in perinuclear vesicles (Fig. 4G), and still colocalized with c-Cbl (Fig. 4I). By contrast, when cells were stimulated for 60 minutes on ice, EGFRs formed clusters at the plasma membrane (Fig. 4J), in which c-Cbl colocalized (Fig. 4L). The clusters were only present on the apical membrane of the cells, as is shown in an *xz* section in Fig. 4L. EGFRs localized on

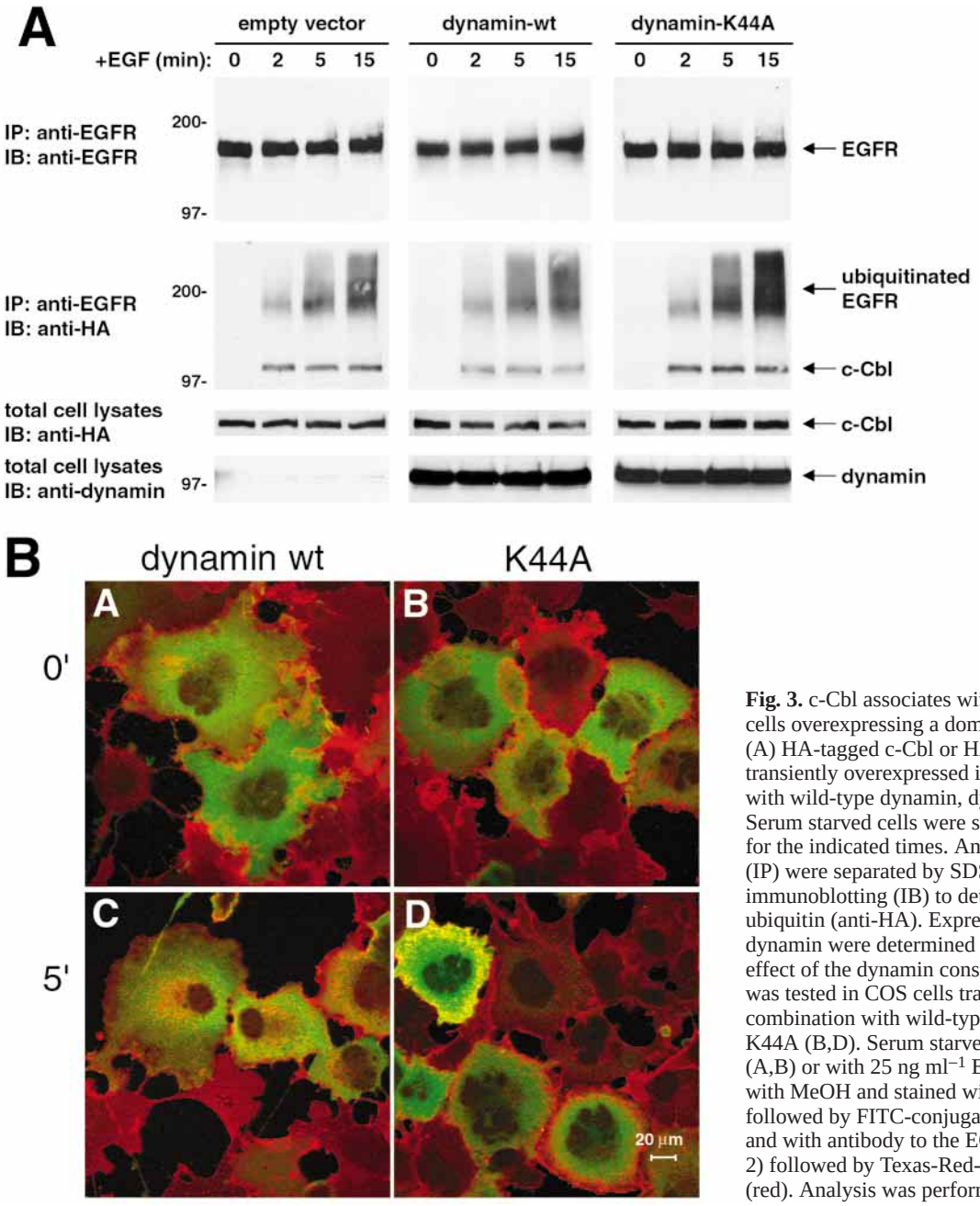

Fig. 3. c-Cbl associates with and ubiquitinates the EGFR in cells overexpressing a dominant negative form of dynamin. (A) HA-tagged c-Cbl or HA-tagged ubiquitin were transiently overexpressed in COS-7 cells in combination with wild-type dynamin, dynamin-K44A or empty vector. Serum starved cells were stimulated with 25 ng ml^{-1} EGF for the indicated times. Anti-EGFR immunoprecipitates (IP) were separated by SDS-PAGE and subjected to immunoblotting (IB) to detect the EGFR, or c-Cbl and ubiquitin (anti-HA). Expression levels of c-Cbl and dynamin were determined in total cell lysates. (B) The effect of the dynamin constructs on EGFR internalization was tested in COS cells transiently expressing c-Cbl in combination with wild-type dynamin (A,C) or dynamin-K44A (B,D). Serum starved cells were incubated without (A,B) or with 25 ng ml^{-1} EGF for 5 minutes (C,D), fixed with MeOH and stained with antibody to dynamin followed by FITC-conjugated secondary antibody (green) and with antibody to the EGFR (polyclonal antibody RK-2) followed by Texas-Red-conjugated secondary antibody (red). Analysis was performed by confocal microscopy.

the basal membrane of the cell were not clustered, nor were they associated with c-Cbl, most probably because they did not come into contact with ligand.

Because treatment of cells with saponin might affect protein distribution in lipid bilayers, we also studied the localization of EGFR and c-Cbl in directly fixed cells. For this purpose, we used a CHO cell line stably overexpressing the human EGFR. In these CHO cells, endogenous c-Cbl can efficiently downregulate the EGFR (data not shown). In the absence of stimulation, EGFRs were present at the plasma membrane (Fig. 5A), whereas endogenous c-Cbl was diffusely distributed in the cytosol (Fig. 5B). Two minutes after EGF stimulation at 37°C, the cell surface displayed punctate staining with both anti-EGFR (Fig. 5D) and anti-c-Cbl (Fig. 5E) antibodies. The merged image shows that EGFR and c-Cbl colocalized in clusters at the cell surface (Fig. 5F). Only a fraction of the c-Cbl molecules appears to be recruited to the activated EGFR, suggesting that c-Cbl is not a limiting factor in EGFR downregulation. This agrees with biochemical analysis of that process in this cell line (not shown). Furthermore, c-Cbl might associate with other tyrosine kinase coupled receptor systems in the cell. After prolonged exposure of the cells to EGF, EGFRs were found in endosomes, together with c-Cbl, as reported earlier (Levkowitz et al., 1998; Lill et al., 2000). In time, stained endosomal structures clustered close to the nucleus. After 60 minutes of stimulation at 37°C, staining was diminished in intensity (Fig. 5G-I). However, in some vesicles, c-Cbl still colocalized with the EGFR at this point (Fig. 5G-I). This observation suggests that c-Cbl stays associated with the EGFR along the endocytic pathway. In cells that were

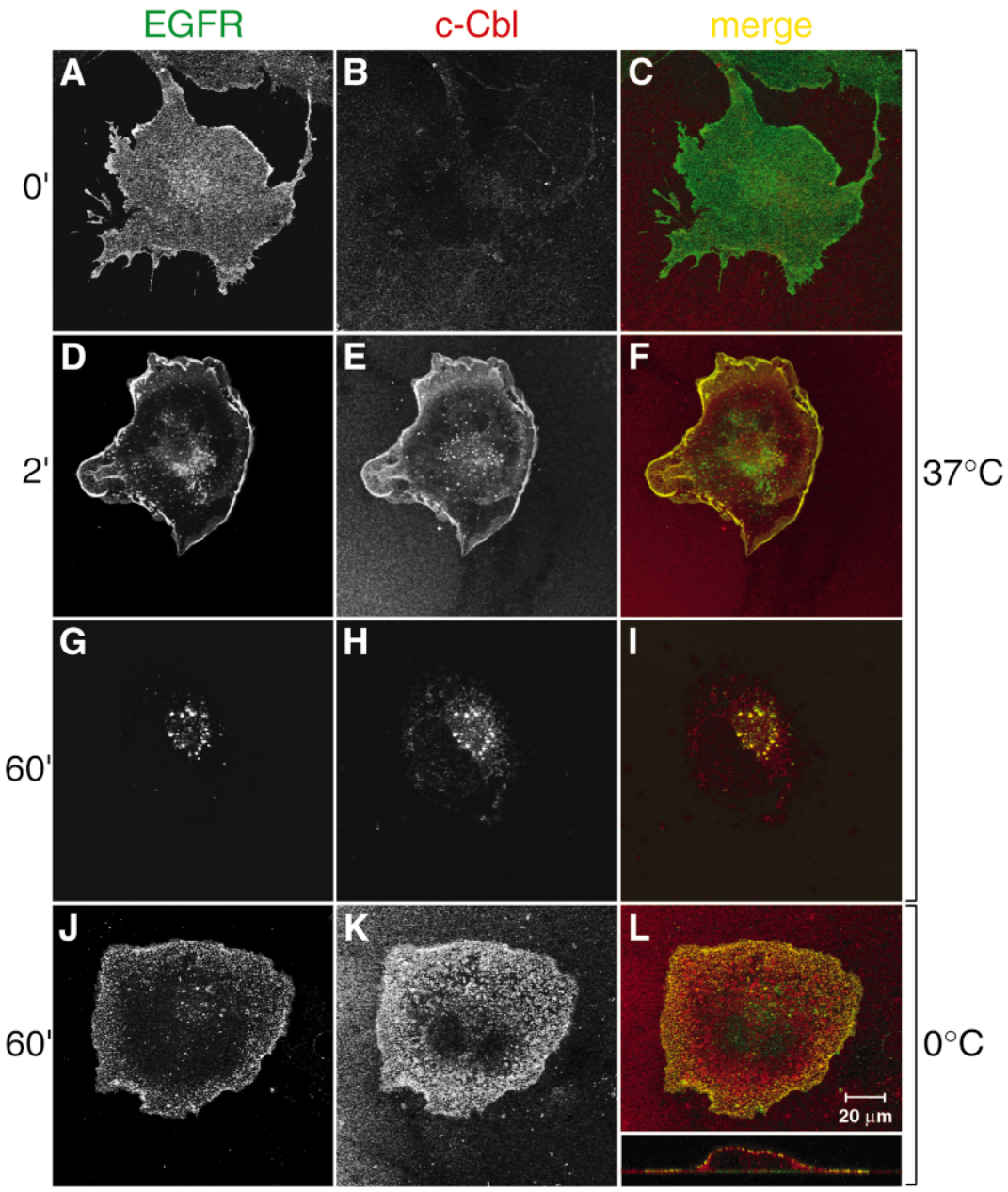

Fig. 4. c-Cbl and the activated EGFR colocalize at the plasma membrane of permeabilized COS cells. COS-7 cells transfected with *c-cbl* cDNA were plated on coverslips, serum starved and stimulated with 25 ng ml^{-1} EGF for the indicated time periods at 37°C (A-I) or on ice (J-L). Prior to fixation, cells were permeabilized with saponin. Cells were stained with mAb directed against the EGFR followed by FITC-conjugated secondary antibody (green; A,D,G,J) and with polyclonal antibody directed against c-Cbl followed by Texas-Red-conjugated secondary antibody (red; B,E,H,K) and analysed by confocal microscopy. Merged images are shown in C, F, I and L. The confocal image in panels A-C was taken at the level of the basal membrane, that in panels G-I at the middle of the cell. The insert in L shows a *xz* section.

stimulated with EGF for 60 minutes on ice, EGFRs remained at the cell surface and organized in clusters in which c-Cbl was colocalized (Fig. 5L). These data confirm the observations obtained in permeabilized COS cells.

EGFR and c-Cbl localization was also studied in CHO cells transfected with wild-type dynamin and the K44A mutant. As shown in Fig. 6 (G-I), c-Cbl colocalized with EGFR in endosomes 5 minutes after EGF stimulation in wild-type-dynamin-overexpressing cells. The dynamin-K44A effectively blocked EGFR internalization (Fig. 6D) and c-Cbl was recruited to the plasma-membrane-localized receptors (Fig. 6E,F). The collective results argue that c-Cbl associates with the activated EGFR at the plasma membrane prior to pinching off of clathrin-coated vesicles, and that c-Cbl remains associated with the EGFR throughout the endocytic route.

c-Cbl and the EGFR colocalize outside and within clathrin-coated pits, and in multivesicular endosomes

Immunogold electron microscopy was used to study the subcellular localization of the EGFR and c-Cbl during endocytosis at high resolution. Because endogenous c-Cbl was difficult to detect with this technique, we used COS cells transiently expressing exogenous c-Cbl, taking care to analyse only those cells that had low expression levels of the protein. In the absence of ligand, the EGFR was mainly found at the cell surface but some membrane-associated gold label was also detected within the cell. c-Cbl was randomly distributed in the cytoplasm and not specifically associated with membrane structures (results not shown). Incubation of cells with EGF for 2-5 minutes at 37°C recruited EGFRs to coated pits (Fig. 7iA). Several coated pits could be identified that stained for c-Cbl

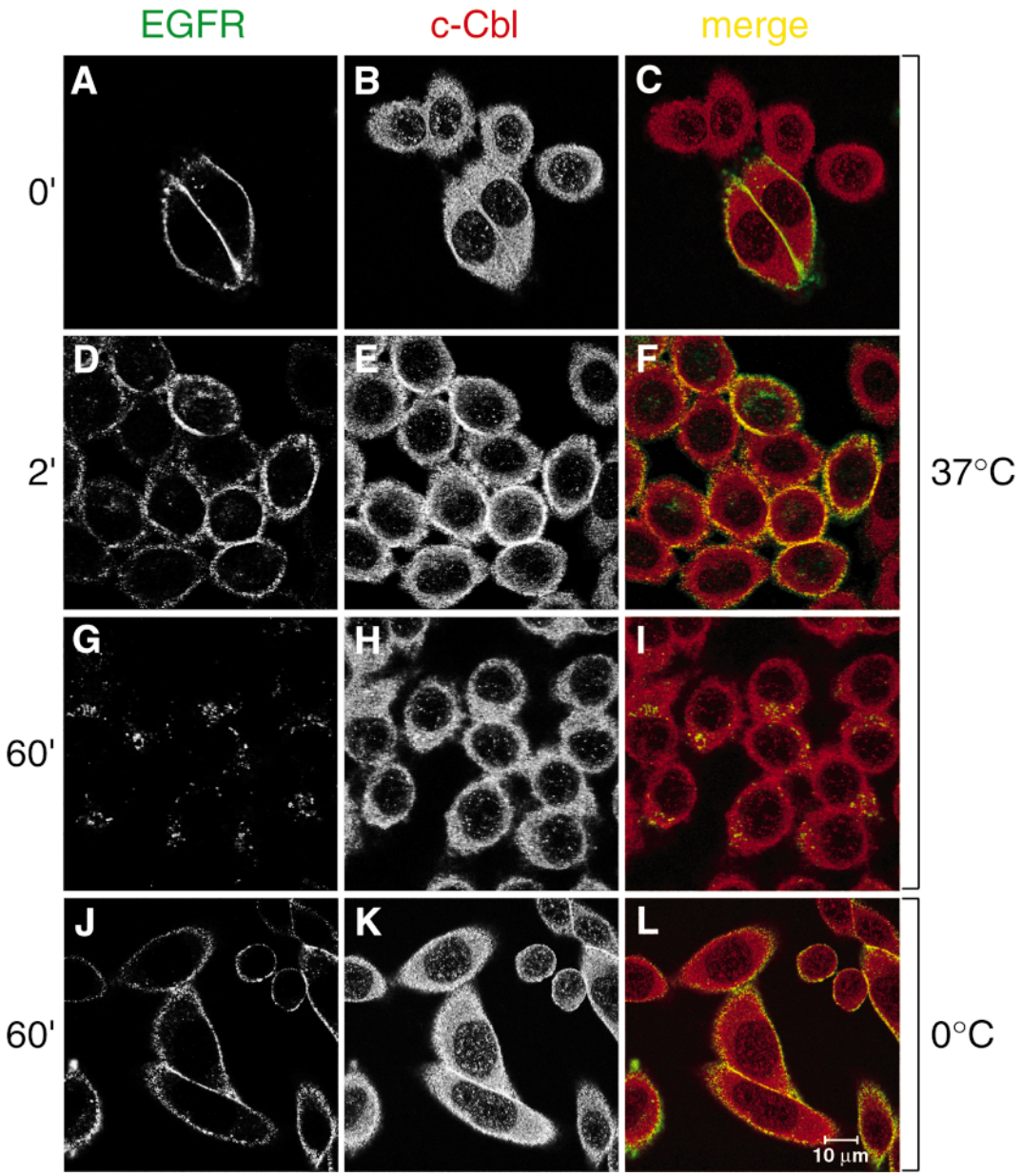

Fig. 5. Endogenous c-Cbl and the activated EGFR colocalize at the plasma membrane of non-permeabilized CHO cells. CHO cells stably expressing the human EGFR were plated on coverslips, serum starved and stimulated with 25 ng ml^{-1} EGF for the indicated time periods at 37°C (A-I) or on ice (J-L). Cells were fixed and stained as indicated for Fig. 4 to detect the EGFR (green; A,D,G,J), or c-Cbl (red; B,E,H,K) by confocal microscopy. Merged images are shown in C, F, I and L. The confocal image in panels G-I was taken at the middle of the cells.

(Fig. 7iB). When cells had been stimulated for 60 minutes on ice, both EGFR (Fig. 7iC) and c-Cbl (Fig. 7iD) could be detected at dense membrane structures, which were often flat and extended. Using antibodies directed against clathrin, we found that these were clathrin-coated (Fig. 7iiB). Double labelling using small and large gold particles clearly identified the presence of c-Cbl at these structures (Fig. 7iiB). c-Cbl localized together with the EGFR at the same coated pits, but also outside these structures (Fig. 7iiA). In a preparation derived from saponin-treated COS cells, which had been stimulated at 37°C, c-Cbl was found at coated vesicles, as well as the plasma membrane (Fig. 7iii).

When cells were stimulated with EGF at 37°C for longer time periods, the EGFR was detected in endosomes, as well as in multivesicular bodies. In the latter structures, the EGFR was found at the surrounding membrane and in internal vesicles (Fig. 8A). Sections incubated with antibody to c-Cbl, revealed labelling around the multivesicular bodies as well as within the internal vesicles (Fig. 8B). By double labelling, c-Cbl was shown to colocalize with the EGFR in the internal vesicles (Fig. 8C). These results demonstrate that c-Cbl associates with the activated EGFR at the plasma membrane both outside and in clathrin-coated pits. The presence of c-Cbl in endosomes and internal vesicles of multivesicular bodies indicates that c-Cbl remains associated with the EGFR throughout the endocytic route.

DISCUSSION

It is known that c-Cbl attenuates the function of protein tyrosine-kinase-coupled receptors by promoting their downregulation (Lee et al., 1999; Levkowitz et al., 1998; Miyake et al., 1998). However, it is not clear which stages of

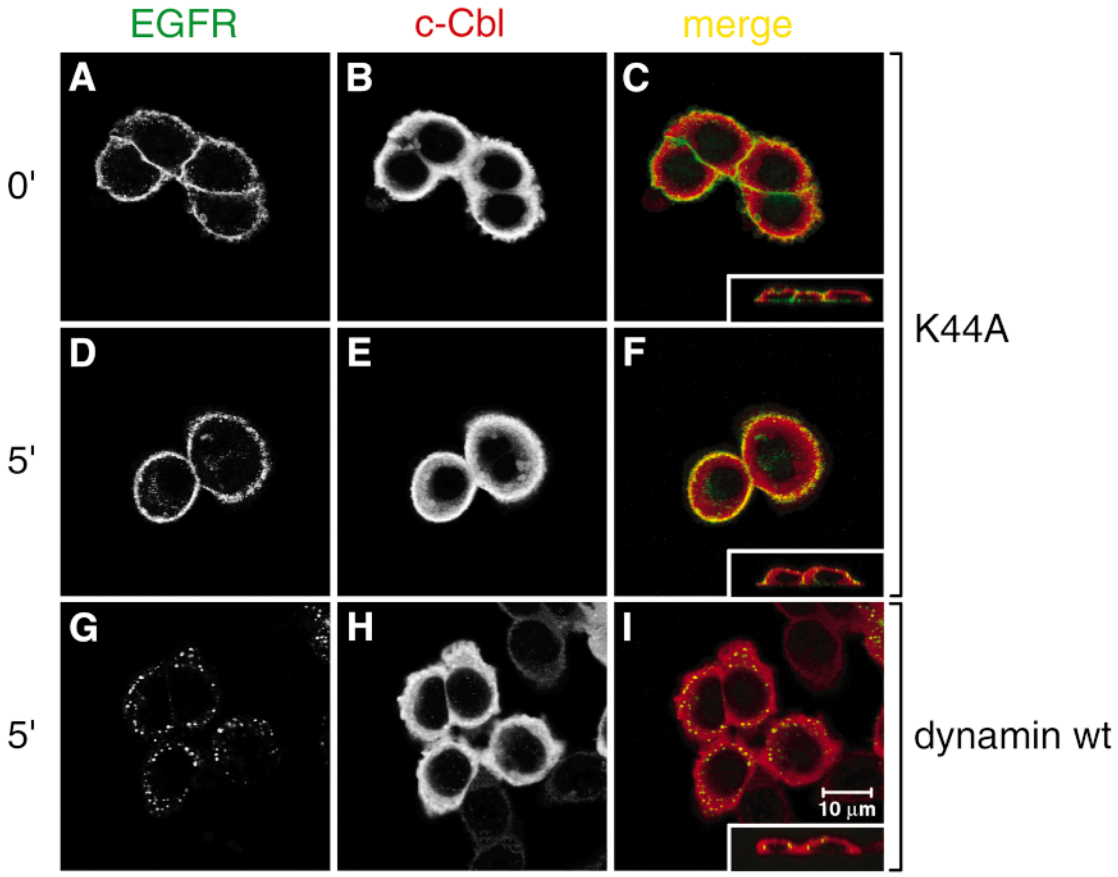

Fig. 6. c-Cbl and the EGFR colocalize at the plasma membrane in the presence of dominant negative dynamin. The human EGFR and c-Cbl were coexpressed in CHO cells, in combination with either the dynamin K44A mutant (A-F) or wild-type dynamin (G-I). Cells were serum starved and incubated without (A-C) or with 25 ng ml^{-1} EGF for 5 minutes (D-I). Cells were fixed and stained with antibodies directed against the EGFR (green; A,D,G) and c-Cbl (red; B,E,H) and analysed by confocal microscopy. Merge images are shown in C, F and I. The confocal image in panels G-I was taken at the middle of the cells. Inserts show *xz* sections.

the endocytic pathway it regulates. We have found by biochemical and confocal analysis that c-Cbl is recruited to the activated EGFR at the plasma membrane. Immunogold electron microscopy revealed that c-Cbl colocalizes with the EGFR both outside and within clathrin-coated pits. These observations point to a role for c-Cbl in the regulation of the early stages of EGFR endocytosis, i.e. receptor recruitment into clathrin-coated pits and/or the formation of clathrin-coated vesicles. Our data are in agreement with those of Wang et al., who found that c-Cbl translocates to the plasma membrane in CSF-1-stimulated macrophages (Wang et al., 1996; Wang et al., 1999). Furthermore, in macrophages from c-$Cbl^{-/-}$ mice, the CSF-1R was not multiply ubiquitinated and was internalized more slowly than in wild-type cells (Lee et al., 1999). Selective and rapid recruitment of ligand-bound EGFRs into clathrin-coated pits requires specific internalization signals in the cytoplasmic tail of the receptor (Sorkin, 1998), a functional receptor kinase domain (Felder et al., 1992; Lamaze and Schmid, 1995; Lund et al., 1990; Wiley et al., 1991) and tyrosine phosphorylation of the cytoplasmic tail (Helin and Beguinot, 1991; Sorkin et al., 1991). These findings are in agreement with a role for c-Cbl in clathrin-dependent receptor endocytosis, because c-Cbl function depends on the same parameters.

Levkovitz et al. (1998), however, described c-Cbl as a resident protein of endosomes, which enhances EGFR downregulation by promoting sorting of the receptor to lysosomes. They demonstrated that, under conditions of v-Cbl overexpression, the EGFR was rescued from sorting to lysosomes and recycled back to the cell surface. v-Cbl did not affect localization of the EGFR in endosomes or decrease the rate of ligand uptake. Therefore, they did not consider the possibility that c-Cbl might play a role in receptor internalization. The discrepancy with our data can be resolved, because Levkovitz et al. used high concentrations of EGF, which might saturate the clathrin-dependent internalization pathway (Wiley, 1988). Under such conditions, EGFR use a ligand- and clathrin-independent internalization pathway (Sorkin, 1998; Watts and Marsh, 1992), which is presumably not regulated by c-Cbl.

Possibly, c-Cbl provides an internalization signal to the ligand-receptor complex. Signals that mediate constitutive binding to clathrin-coated pits include tyrosine-based motifs and the dileucine motif (Sorkin, 1998). The motif YXXΦ (X is any amino acid, Φ has a bulky hydrophobic group), which is present in, for instance, the transferrin receptor, provides a binding site for the adaptor protein AP-2, a heterotetrameric protein that induces the assembly of clathrin triskelions at the plasma membrane. The EGFR can interact directly with AP-2 via the tyrosine-based motif FYRAL (Sorkin et al., 1996). Whereas transferrin receptor internalization was found to be dependent on the residues in AP-2 that interact with the tyrosine motif, ligand-induced EGFR endocytosis was not (Nesterov et al., 1999). Therefore, clathrin-mediated internalization of the EGFR must depend on another signal. We suggest that c-Cbl-mediated ubiquitination of the EGFR provides such a signal. We have demonstrated that c-Cbl ubiquitinates the EGFR prior to its internalization. In agreement with our findings, the EGFR was recently found to

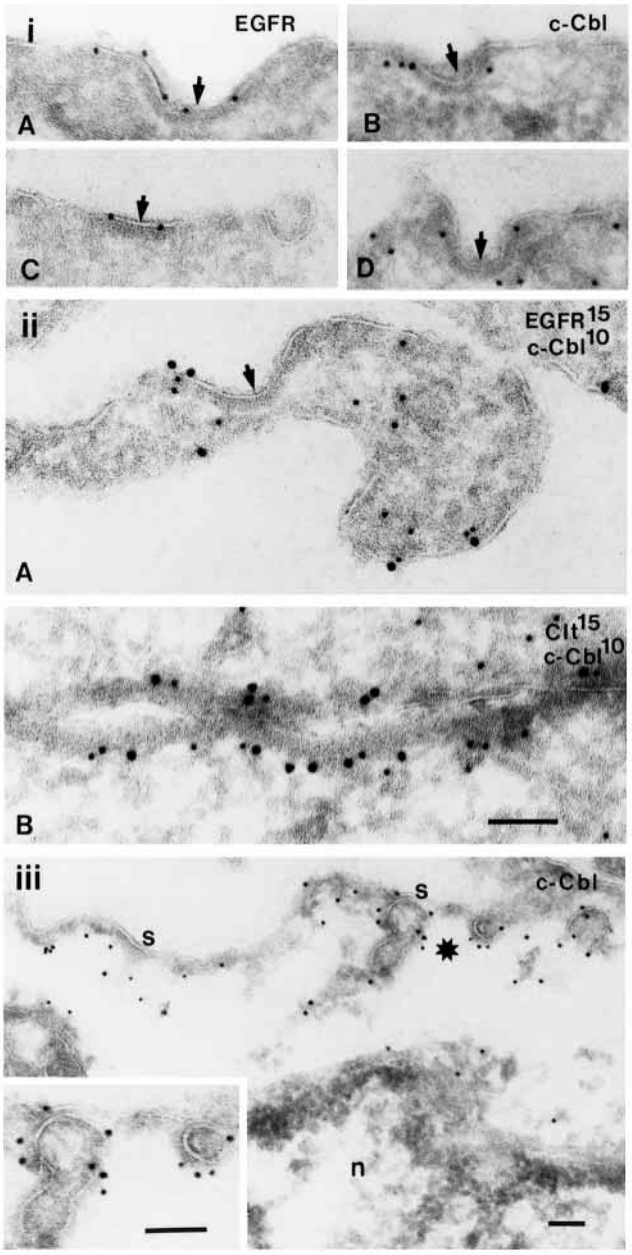

Fig. 7. Immuno-electron microscopy reveals localization of c-Cbl and EGFR outside, and at clathrin-coated pits. COS-7 cells transfected with *c-cbl* cDNA were stimulated with EGF for 2 minutes at 37°C (iA,iB), for 60 minutes at 0°C (iC,iD,ii) or for 5 minutes at 37°C, followed by treatment for 15 minutes with saponin on ice (iii). All preparations were fixed and ultrathin cryosections were prepared for immunogold labelling. Sections were single labelled with antibodies to EGFR (iA,iC) or c-Cbl (iB,iD,iii), or double labelled with antibodies to EGFR and c-Cbl (iiA) or antibodies to clathrin and c-Cbl (iiB). Abbreviations: c-Cbl^{10}, c-Cbl 10 nm gold particle; Clt^{15}, clathrin 15 nm gold particle; EGFR^{15}, EGFR 15 nm gold particle; N, the nucleus; S, cell surface. Inset in iii is a higher magnification of the marked area (asterisk) showing c-Cbl on coated vesicles. Magnification: i and ii show the same magnification. Bar, 100 nm.

be ubiquitinated in Hela cells overexpressing K44A dynamin (Stang et al., 2000).

There is ample evidence from the yeast system that ubiquitination serves a role in receptor endocytosis. A chimeric receptor consisting of a single ubiquitin fused to a cytoplasmic tail-truncated Ste2p receptor was effectively internalized. Apparently, ubiquitin sufficed as an internalization signal in this case (Shih et al., 2000). Whereas monoubiquitination is sufficient (Terrell et al., 1998), multiple ubiquitination increased the internalization rate of the Ste3p receptor (Roth and Davis, 2000). In case of the yeast uracil permease, lysine 63-linked diubiquitination was needed to obtain maximal internalization rates (Galan and Haguenauer-Tsapis, 1997). Similar to the findings in yeast, a single ubiquitin molecule fused to the cytoplasmic region of the invariant chain or the interleukin-2 receptor α chain was sufficient to mediate receptor internalization in Hela cells (Nakatsu et al., 2000). Moreover, plasma membrane levels of an ubiquitination-defective mutant of the epithelial sodium channel (ENaC) were increased (Staub et al., 1997). In case of the human growth hormone receptor, interaction with components of the ubiquitin-conjugating machinery is required for its endocytosis, whereas receptor ubiquitination does not seem to be involved (Govers et al., 1999; Strous et al., 1996).

Several other molecules have been implicated in recruitment of the EGFR into clathrin-coated pits, such as Grb-2, Eps15 and c-Src. The Grb-2 adaptor protein can bind with its SH2 domain to the cytoplasmic tail of the EGFR, whereas its SH3 domains can interact with dynamin (Seedorf et al., 1994; Wang and Moran, 1996). Overexpression of the Grb-2 SH2 domain was found to inhibit EGFR internalization (Wang and Moran, 1996). Eps15 and Eps15R are substrates of the activated EGFR and are essential for receptor internalization (Carbone et al., 1997). Tyrosine phosphorylation of Eps15 is required for ligand-induced but not for constitutive endocytosis (Confalonieri et al., 2000). Eps15 interacts with AP-2 and clathrin but does not bind directly to the EGFR, implying the involvement of other components (van Delft et al., 1997). The protein tyrosine kinase c-Src, which is activated by the EGFR, has been implicated in the exit of the activated EGFR from caveolae, as well as its internalization via clathrin-coated pits (Carpenter, 2000; Ware et al., 1997). c-Src phosphorylates the clathrin heavy chain and might thus control the assembly of a clathrin network (Wilde et al., 1999).

c-Cbl can easily be visualized as intermediate in Grb-2- and Src-regulated receptor endocytosis, because both molecules can interact via their SH3 domains with the proline-rich region in the C-terminus of c-Cbl. Furthermore, phosphorylated tyrosine residues in the C-terminus serve as docking sites for the SH2 domains of Vav, p85 and the adaptor protein Crk (Lupher et al., 1999; Smit and Borst, 1997; Thien and Langdon, 1998). It has been hypothesized that c-Cbl interacts with Eps15 via Crk (Smit and Borst, 1997), thus providing the link between Eps15 and the EGFR. Together, these data suggest that the C-terminal region of c-Cbl provides additional cues for receptor internalization.

Our biochemical and confocal data showed a prolonged association of c-Cbl with the activated EGFR. Immunogold electron microscopy allowed detection of c-Cbl in the internal vesicles of multivesicular bodies. We conclude that the c-Cbl-EGFR interaction is maintained throughout the endocytic pathway. According to the model of Futter et al. (1996), recycling receptors are lost from maturing multivesicular

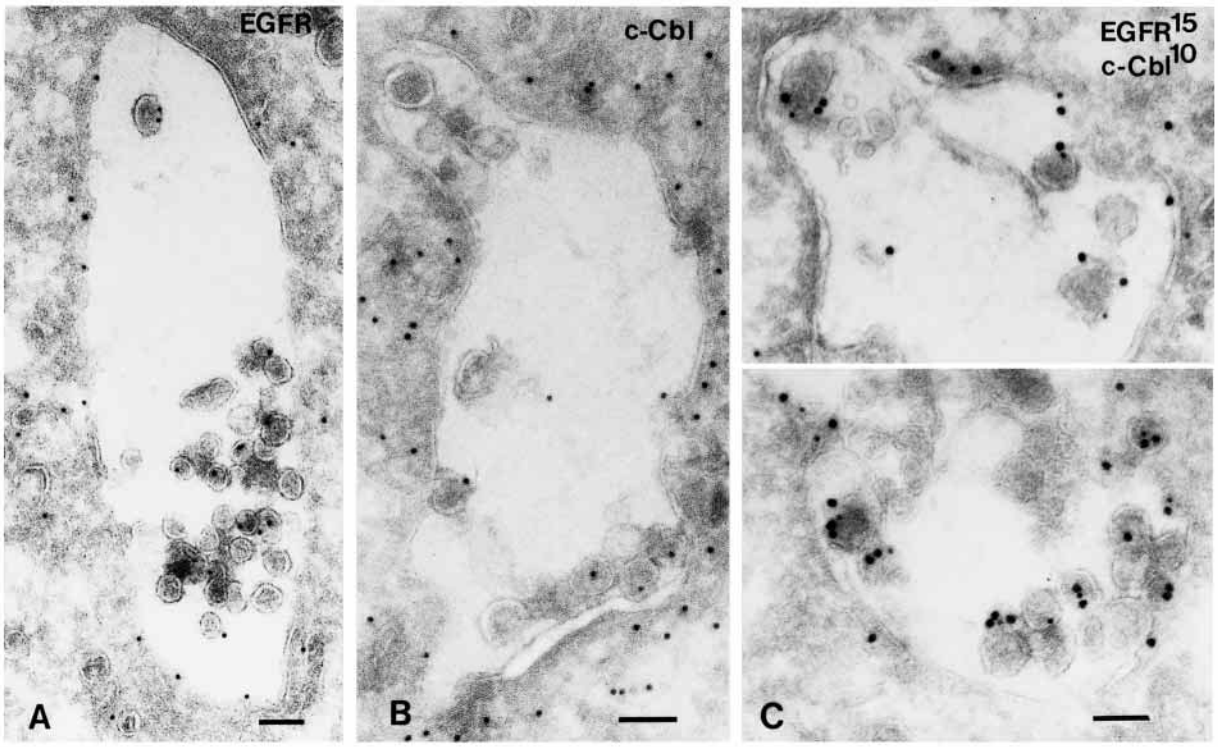

Fig. 8. Immuno-electron microscopy reveals c-Cbl in multivesicular bodies. COS-7 cells transfected with *c-cbl* cDNA were stimulated with EGF for 15 minutes (A,B) or 60 minutes at 37°C (C). Ultrathin cryosections were single labelled to detect EGFR (A) or c-Cbl (B), or double labelled to detect both EGFR and c-Cbl (C). Abbreviations: c-Cbl^{10}, c-Cbl 10 nm gold particle; EGFR^{15}, EGFR 15 nm gold particle. Bar, 100 nm.

bodies, whereas the EGFR becomes localized in internal vesicles and ends up in lysosomes upon fusion of both organelles. This suggests that c-Cbl will be degraded together with the EGFR in lysosomes. We find a small decrease in c-Cbl levels in total lysates of stimulated COS cells in time (Fig. 1). Others have found no evidence for degradation of c-Cbl upon activation of the CSF-1R and describe that c-Cbl is relocated to the cytosol after receptor internalization (Wang et al., 1996). These differences might be receptor specific.

We propose a model in which c-Cbl-mediated ubiquitination facilitates recruitment of activated EGFRs into clathrin-coated pits by providing a ligand-dependent internalization signal. In this way, c-Cbl enhances internalization of the ligand-receptor complex via the saturatable, clathrin-mediated endocytosis pathway. Subsequently, c-Cbl might be involved in receptor sorting and finally is routed, together with the EGFR, towards the lysosomes.

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