

Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies

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EGF, but not TGF α , efficiently induces degradation of the EGF receptor (EGFR). We show that EGFR was initially polyubiquitinated to the same extent upon incubation with EGF and TGF α , whereas the ubiquitination was more sustained by incubation with EGF than with TGF α . Consistently, the ubiquitin ligase c-Cbl was recruited to the plasma membrane upon activation of the EGFR with EGF and TGF α , but localized to endosomes only upon activation with EGF. EGF remains bound to the EGFR upon endocytosis, whereas TGF α dissociates from the EGFR. Therefore, the sustained polyubiquitination is explained by EGF securing the kinase activity of endocytosed EGFR. Overexpression of the dominant negative N-Cbl inhibited

ubiquitination of the EGFR and degradation of EGF and EGFR. This demonstrates that EGF-induced ubiquitination of the EGFR as such is important for lysosomal sorting. Both lysosomal and proteasomal inhibitors blocked degradation of EGF and EGFR, and proteasomal inhibitors inhibited translocation of activated EGFR from the outer limiting membrane to inner membranes of multivesicular bodies (MVBs). Therefore, lysosomal sorting of kinase active EGFR is regulated by proteasomal activity. Immuno-EM showed the localization of intact EGFR on internal membranes of MVBs. This demonstrates that the EGFR as such is not the proteasomal target.

Introduction

Several transmembrane proteins are ubiquitinated upon ligation (for reviews see Bonifacino and Weissman, 1998; Hicke, 1999). The EGF receptor (EGFR)* is polyubiquitinated upon activation of the EGFR intrinsic kinase activity (Galcheva-Gargova et al., 1995). c-Cbl was recently demonstrated to possess ubiquitin ligase activity (Joazeiro et al., 1999), and it was further reported that the NH₂-terminal region of c-Cbl, containing the RING finger responsible for the ubiquitin ligase activity (Levkowitz et al., 1999), was sufficient to enhance EGFR downregulation (Lill et al., 2000). c-Cbl has also been demonstrated to negatively regulate PDGF receptor (PDGFR)-dependent cell proliferation by ligand-dependent ubiquitination and subsequent degradation of both PDGFR α and PDGFR β (Miyake et al., 1998, 1999).

In accordance with these findings, it has been suggested that ubiquitination marks receptors for degradation (Hicke, 1997; Heldin et al., 1998; Levkowitz et al., 1998).

The yeast G protein-coupled receptor Ste2p is accumulated in high molecular weight ubiquitinated forms at the cell surface in response to the binding of α -factor in endocytosis-deficient yeast cells (Hicke and Riezman, 1996). This finding established a link between ligand-induced ubiquitination, endocytosis, and eventual targeting for vacuolar degradation in yeast. It has also been reported that the ubiquitin conjugation system is required for ligand-induced endocytosis of the growth hormone receptor (van Kerkhof et al., 2000). Based on these and other observations, the ubiquitin system has been suggested as an important regulatory system for clathrin-dependent endocytosis (Strous and Govers, 1999). This is in accordance with the demonstration that polyubiquitination of the EGFR occurs at the plasma membrane. We demonstrated that in endocytosis-deficient cells the EGFR was transiently polyubiquitinated, but not degraded (Stang et al., 2000).

It is still unclear to what extent proteasomal processing is involved in downregulation of receptors. In the case of the PDGFR, the degradation was reportedly inhibited <20%

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*Abbreviations used in this paper: CHX, cycloheximide; EGFR, EGF receptor; GFP, green fluorescent protein; HA, hemagglutinin; MVBs, multivesicular bodies; PDGFR, PDGF receptor; Tf, transferrin.

Key words: EGF; c-Cbl; ubiquitination; proteasome; multivesicular bodies

by proteasome inhibitors (Mori et al., 1995a, 1995b). The receptor tyrosine kinase c-Met consists of a 50-kD extracellular α subunit and a 140-kD membrane-spanning β subunit. Upon ligand binding, the β subunit was ubiquitinated and subsequently degraded in a proteasome-dependent manner (Jeffers et al., 1997). Also in the case of ErbB2, proteasome inhibitors have been demonstrated to inhibit degradation (Mimnaugh et al., 1996). However, the question of whether or not ErbB2 is in fact endocytosed is still controversial. It was recently reported that the antibiotic geldanamycin induced degradation of ErbB2 and that degradation of the COOH-terminal fragment was prevented by proteasome inhibitors, whereas degradation of the membrane-anchored 135-kD fragment was blocked by inhibitors of the endocytosis-dependent degradation pathway (Tikhomirov and Carpenter, 2000). Therefore, the functional role of ubiquitination in endocytosis and downregulation of receptors is unresolved and challenging.

It has been established that EGF and TGF α differ in their ability to induce processing of the EGFR (Ebner and Derynck, 1991). EGF is a more acidic molecule than is TGF α and remains bound to the EGFR upon internalization during acidification of the endocytic vesicle, finally resulting in proteolytic degradation of both ligand and receptor in lysosomes. In contrast, TGF α rapidly dissociates from the receptor upon internalization, resulting in recycling of EGFR to the cell surface (Sorkin and Waters, 1993). We report here that in spite of differential degradation induced by EGF and TGF α , both ligands equally efficiently induce initial ubiquitination and endocytosis of the EGFR. However, EGF, which in contrast to TGF α secures the kinase activity of the EGFR upon endocytosis, maintains a sustained polyubiquitination of the EGFR. Consistently, colocalization of EGFR and c-Cbl on endosomes is observed only upon incubation with EGF. By overexpression of the dominant negative N-Cbl, we confirmed that Cbl-induced ubiquitination is important for degradation of EGF-bound EGFR, but not for endocytosis. By the use of inhibitors of endosomal/lysosomal and proteasomal degradation, we further found that degradation of EGFR and EGF requires proteasomal processing. Therefore, we submit that ubiquitination per se is not a signal for EGFR degradation. However, sustained ubiquitination is required for efficient sorting of the EGFR to a degrading compartment, and proteasomal activity is required for efficient transport of EGF and EGFR to inner membranes of MVBs.

Results

EGFR is efficiently degraded upon incubation with EGF, but not with TGF α

To confirm differential degradation of the EGFR upon incubation with EGF compared with TGF α (Ebner and Derynck, 1991), Hep2 cells were incubated with EGF and TGF α in the presence of cycloheximide (CHX), facilitating studies of EGFR degradation by inhibiting protein synthesis. Immunoblotting showed that there was a visible degradation of EGFR already upon incubation with EGF for 2 h (Fig. 1 A, top). In contrast, there was only a slight degradation of the EGFR upon incubation with TGF α for as long

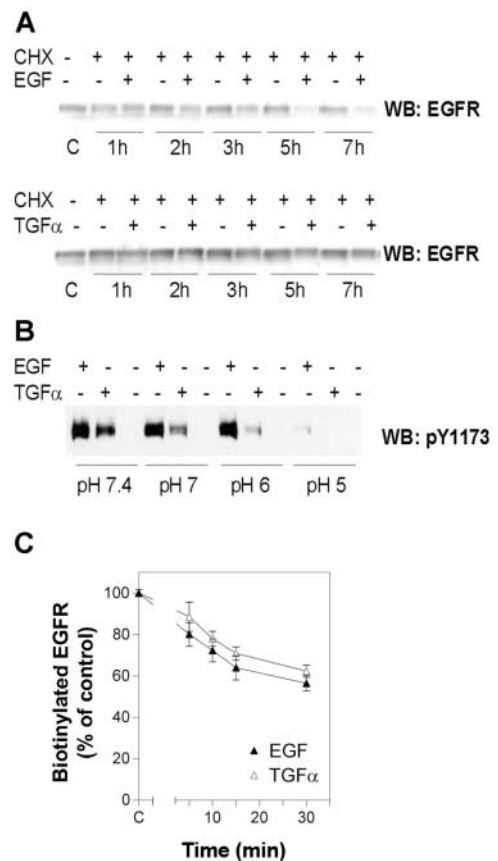


Figure 1. Degradation and pH dependent phosphorylation of the EGFR upon incubation with EGF and TGF α . (A) Hep2 cells were incubated with CHX (25 μ g/ml) and EGF (10 nM) (top) or TGF α (10 nM) (bottom) at 37°C for the indicated time periods. The cells were lysed and subjected to SDS-PAGE and immunoblotting with antibody to EGFR (Stang et al., 2000). c, control cells not incubated with ligand nor CHX. (B) Hep2 cells were incubated with EGF (10 nM) or TGF α (10 nM) on ice for 15 min. The medium was removed, and the cells were incubated on ice for 20 min in MEM without bicarbonate with BSA and MES (10 mM) adjusted to pH 5.0, 6.0, 7.0 and 7.4, containing EGF (10 nM) or TGF α (10 nM). The cells were lysed and subjected to SDS-PAGE and immunoblotting with antibody to activated EGFR (pY1173). (C) Hep2 cells were incubated with 10 nM EGF (\blacktriangle) or 10 nM TGF α (\triangle) at 37°C for indicated times, before the cells were biotinylated on ice. Biotinylated EGFR was immunoprecipitated and analyzed by SDS-PAGE and immunoblotting. Biotinylated EGFR was normalized for precipitation efficiency using total EGFR immunoprecipitated. The data represent the mean of four independent experiments \pm SEM.

as 7 h (Fig. 1 A, bottom). pH decreases gradually in endocytic vesicles. Whereas intravesicular pH is \sim 6.0 in early endosomes, the pH of lysosomes is 4.5–5.0 (Yamashiro and Maxfield, 1984). The EGFR is fully phosphorylated on ice (unpublished data). However, the phosphorylation is pH sensitive (Fig. 1 B). In cells incubated with TGF α , the EGFR became dephosphorylated upon exposure to slightly reduced pH. The EGFR in cells incubated with EGF was dephosphorylated only at pH values similar to those found in lysosomes. These results can be explained by the fact that TGF α is released from the EGFR at slightly reduced pH values, whereas EGF is not. Accordingly, TGF α will be released in early endosomes, allowing the EGFR to recycle to

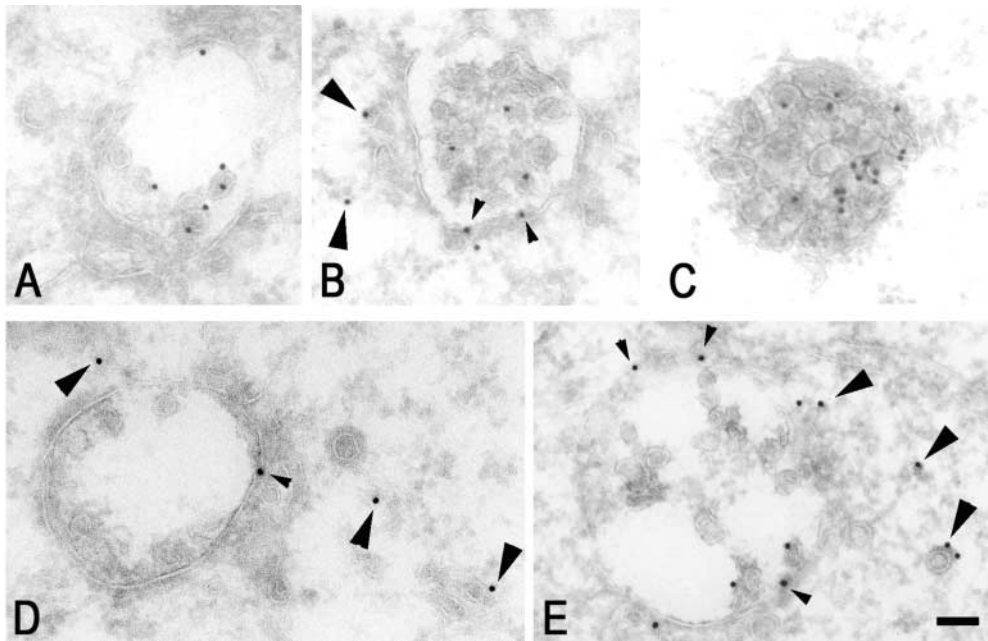


Figure 2. The EGFR shows different localization within MVBs upon incubation with EGF compared with TGF α . Hep2 cells incubated with EGF (10 nM) (A–C) or TGF α (10 nM) (D–E) on ice for 15 min and chased at 37°C for 30 min were processed for immuno-EM, sectioned and labeled using antibody to EGF (A) or EGFR (B–E). Upon activation with EGF, labeling for EGF (A), as well as for EGFR (B–C), is found on MVBs at all stages of formation. The labeling on MVBs is found on the limiting membrane (small arrowheads), as well as on internal vesicles. In MVBs with a high number of internal vesicles (B and C), labeling is concentrated on the inner membranes. Some labeling for the EGFR is also found on small vesicles within the cytoplasm (large arrowheads). In cells activated with TGF α (D–E), labeling intensity is generally reduced (D), and labeling is mainly localized to the outer limiting membrane of MVBs. Note that the labeling of small vesicles within the cytoplasm appears to be most frequent in TGF α -treated cells. Bar, 100 nm.

the plasma membrane. However, EGF will remain complexed to the EGFR securing EGFR kinase activity and thereby lysosomal sorting. The efficiency of endocytosis induced by EGF and TGF α was assessed by measuring the amount of surface-localized (noninternalized) EGFR by biotinylation. As demonstrated in Fig. 1 C, and as previously reported (Decker, 1990; Ebner and Derynck, 1991), EGF and TGF α induced endocytosis with the same efficiency.

The difference in intracellular trafficking of the EGFR upon activation with EGF versus TGF α , was further studied using immuno-EM. Labeling of thawed cryosections showed that the early events in endocytosis were identical upon stimulation with EGF and TGF α . Upon binding of ligand to cells on ice and subsequent chase at 37°C, the EGFR was internalized via clathrin coated pits. After a 10-min chase, the labeling was found in compartments with tubulovesicular morphology typical of early endosomes (unpublished data). However, after a 30-min chase, the intracellular labeling pattern and intensity showed ligand-dependent differences. Whereas with both ligands labeling was found associated with MVBs (Fig. 2), the morphology of the EGFR-positive MVBs as well as the localization of the EGFR within MVBs varied. In cells incubated with TGF α , labeling intensity was low and mainly found on MVBs containing only a few internal vesicles (Fig. 2, D–E). This possibly represents MVBs at an early stage of formation, and the labeling was largely restricted to the outer, limiting membrane of the MVBs. However, in cells incubated with EGF, labeling was found on MVBs at all stages of formation, and labeling for both EGF and the EGFR was concentrated on

the internal vesicles (Fig. 2, A–C). Quantification of EGFR labeling showed that whereas in cells incubated with TGF α only 25% of the labeling was localized to inner membranes, >80% showed this localization upon incubation with EGF. A similar difference in trafficking has previously been shown between kinase active and kinase inactive EGFR (Felder et al., 1990), demonstrating that the EGFR kinase activity is needed for sorting of the EGFR to internal vesicles within MVBs. In addition to labeling on MVBs, some EGFR was found within small vesicles close to the MVBs. The nature of these vesicles is unknown. However, as this labeling appeared to be most frequent in cells incubated with TGF α , the vesicles might be involved in recycling of the EGFR. Recycling is probably also the reason why intracellular labeling intensity was low upon 30 min chase upon incubation of cells with TGF α . The different labeling found in our experiments probably reflects the different physiological character of EGF and TGF α as EGFR ligands. EGF remains bound to the EGFR, keeping the kinase active within MVBs, whereas TGF α is dissociated in MVBs, and the EGFR kinase is no longer active (Fig. 1 B).

Both EGF and TGF α induce polyubiquitination of EGFR, but the ubiquitination is more sustained with EGF than with TGF α

Ubiquitination of the EGFR was studied by immunoprecipitating conjugated ubiquitin and immunoblotting the immunoprecipitated material using an antibody to EGFR. As demonstrated in Fig. 3 A, both EGF and TGF α induced ubiquitination of the EGFR. However, the amount and af-

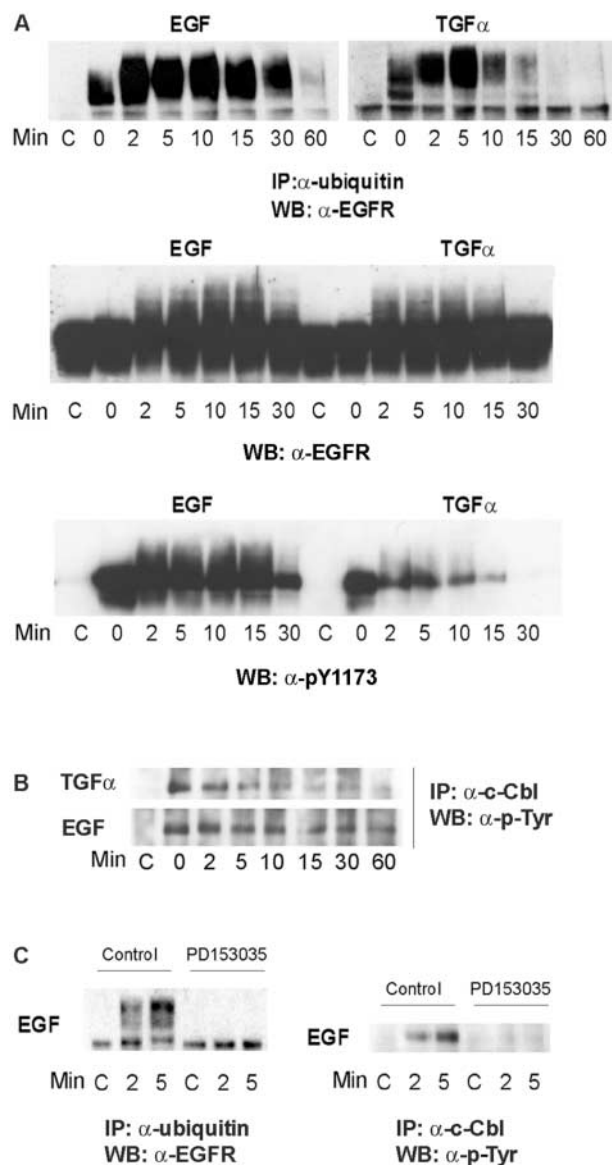


Figure 3. Ubiquitination and phosphorylation of the EGFR and tyrosine phosphorylation of c-Cbl induced by EGF and TGF α . (A) Hep2 cells were incubated with EGF (10 nM) or TGF α (10 nM) on ice for 15 min and chased at 37°C for indicated times. The cells were lysed, and the lysates were subjected to immunoprecipitation with antibody to conjugated ubiquitin, followed by immunoblotting with antibody to EGFR (top). Lysates were also subjected to SDS-PAGE and immunoblotting with antibody to EGFR (middle) or activated EGFR (pY1173) (bottom). c, control cells not incubated with ligand. (B) Hep2 cells were incubated as in (A), and c-Cbl was immunoprecipitated. The precipitated material was subjected to SDS-PAGE and immunoblotting with antibody to phosphotyrosine (pTyr). c, control cells not incubated with ligand. (C) Hep2 cells were preincubated with PD153035 (100 nM) for 2 h at 37°C, before incubation with EGF (10 nM) on ice for 15 min. The cells were subsequently chased in MEM without bicarbonate containing PD153035 (100 nM) at 37°C for the indicated time periods. Conjugated ubiquitin was immunoprecipitated, and the precipitated material was subjected to SDS-PAGE and immunoblotting with antibody to EGFR (left). c-Cbl was immunoprecipitated, and the precipitated material was subjected to SDS-PAGE and immunoblotting with antibody to phosphotyrosine (pTyr) (right).

finity of the antibody used could potentially be a limiting factor with respect to immunoprecipitation of the protein of interest. To exclude the possibility that EGF induces a stronger initial ubiquitination than does TGF α , cell lysates were subjected to SDS-PAGE and immunoblotted with antibody to EGFR. By overexposing the film, we clearly observed polyubiquitination of the EGFR as “upsmeared.” As demonstrated in Fig. 3 A, top and middle, both EGF and TGF α induced the same initial ubiquitination of EGFR. However, the ubiquitination induced by EGF was more sustained than the ubiquitination induced by TGF α . EGF also induced a more sustained phosphorylation of EGFR than did TGF α (Fig. 3 A, bottom). Interestingly, ubiquitination of the EGFR occurred even at 0°C, before endocytosis of EGFR (Fig. 3 A, top).

c-Cbl is the ubiquitin ligase responsible for the EGF-mediated ubiquitination of the EGFR (Waterman et al., 1999; Yokouchi et al., 1999). Therefore, we investigated activation of c-Cbl in Hep2 cells upon EGF- and TGF α -induced activation of EGFR. Immunoblotting of c-Cbl immunoprecipitates with antibody to phosphotyrosine showed that c-Cbl was tyrosine phosphorylated upon incubation both with EGF and with TGF α on ice for 15 min and remained phosphorylated during chase at 37°C for 30–60 min (Fig. 3 B). To confirm that the EGF/TGF α -induced ubiquitination of the EGFR is dependent upon EGFR kinase activity, Hep2 cells were preincubated with the EGFR kinase inhibitor PD153035 for 2 h at 37°C, before incubation with EGF. PD153035 efficiently inhibited ubiquitination of the EGFR (Fig. 3 C, left) and the tyrosine phosphorylation of c-Cbl (Fig. 3 C, right).

TGF α and EGF recruit c-Cbl to the plasma membrane, whereas only EGF recruits c-Cbl to endosomes

Immunocytochemical labeling for confocal microscopy was performed, using antibodies to EGFR and c-Cbl. The specificity of the antibodies was investigated by Western blotting (unpublished data) and further confirmed by ligand-induced changes in localization of EGFR and c-Cbl. The EGFR was mainly localized to the plasma membrane in control cells (Fig. 4 A) and in cells incubated with EGF or TGF α on ice (Fig. 4, D and G). Whereas c-Cbl in control cells mainly localized to the cytoplasm (Fig. 4 B), c-Cbl was recruited to the plasma membrane upon incubation both with EGF and with TGF α on ice (Fig. 4, E and H) and appeared to colocalize with the EGFR (Fig. 4, F and I). In cells incubated with EGF, both the EGFR and c-Cbl were redistributed and colocalized on vesicular structures upon chase at 37°C for 15 min (Fig. 4, J–L). Upon binding of TGF α and further chase at 37°C for 15 min, the EGFR localized both to the plasma membrane and to vesicular structures (Fig. 4 M). However, c-Cbl, relocated to the cytoplasm (Fig. 4 N), and there was no clear colocalization of EGFR and c-Cbl (Fig. 4 O).

Dominant negative Cbl (N-Cbl) inhibits degradation, but not endocytosis of EGF and EGFR

Ubiquitination of the EGFR at the plasma membrane could be important for endocytosis of the EGFR. To address this, we transiently transfected COS-1 cells with a plasmid en-

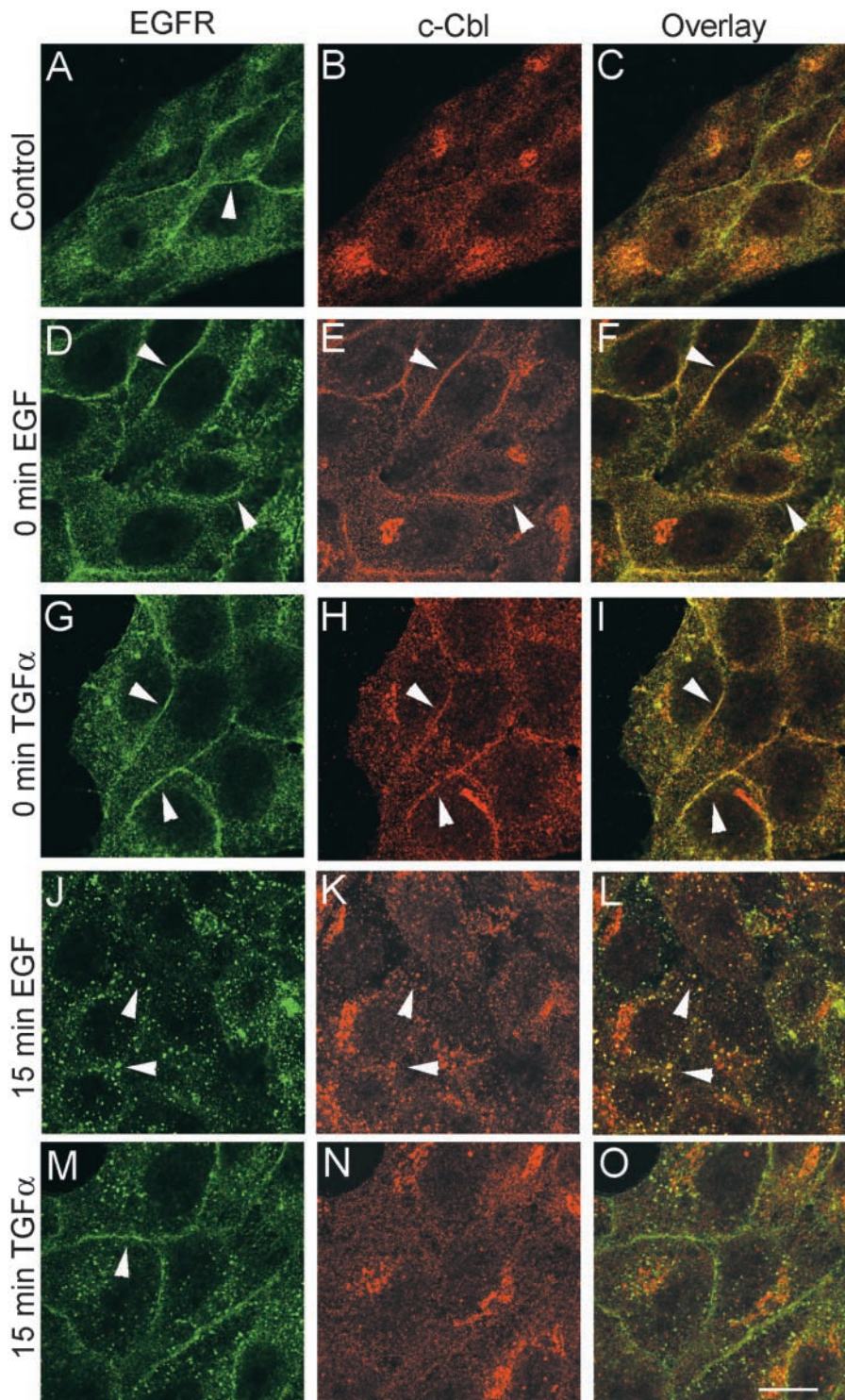


Figure 4. Localization of EGFR and c-Cbl upon incubation with EGF and TGF α . Hep2 cells were incubated without ligand (A–C) or with EGF (10 nM) (D–F and J–L) or TGF α (10 nM) (G–I and M–O) on ice for 15 min. In J–O, the cells were further chased at 37°C for 15 min. The cells were processed and immunostained, as described in Materials and methods, using sheep anti-EGFR (1:500) and rabbit anti-c-Cbl (1:500), followed by CyTM2-conjugated donkey anti-sheep (1:250) (green) and Alexa Fluor 594-conjugated goat anti-rabbit (1:1,000) (red). In nonstimulated cells the EGFR is concentrated at the plasma membrane (A, arrowhead), whereas c-Cbl is localized to the cytoplasm. Upon incubation with EGF or TGF α on ice, c-Cbl is recruited to the plasma membrane and colocalizes with EGFR (D–I, arrowheads). The EGFR colocalizes with c-Cbl in vesicular compartments upon incubation with EGF and chase at 37°C for 15 min, as indicated by arrowheads in J–L. After incubation with TGF α and chase at 37°C for 15 min the EGFR is localized at the plasma membrane, and c-Cbl is localized to the cytoplasm, giving no colocalization. Bar, 50 μ m.

coding HA-tagged N-Cbl. N-Cbl consists of the NH₂-terminal 357 amino acids of c-Cbl, comprising the SH2 domain, but not the RING finger domain responsible for EGFR ubiquitination. When overexpressed, N-Cbl will bind to phosphorylated Tyr 1045 of the EGFR and inhibit binding of c-Cbl, thereby inhibiting EGFR ubiquitination. Transfected and nontransfected cells were compared with respect to internalization of Rh-EGF. As demonstrated in Fig. 5 A, cells overexpressing N-Cbl (identified with anti-

body to hemagglutinin [HA]) internalized Rh-EGF as efficiently as did nontransfected cells. The rate of internalization and degradation of EGF was further studied in COS-1 cells transfected with the plasmid encoding HA-tagged N-Cbl. As demonstrated in Fig. 5 B, the internalization of ¹²⁵I-EGF (0.2 nM) was not altered upon transfection with the N-Cbl plasmid. Degradation and recycling was investigated upon loading transfected cells with ¹²⁵I-EGF (8.5 nM). Degradation was clearly inhibited, and recycling was in-

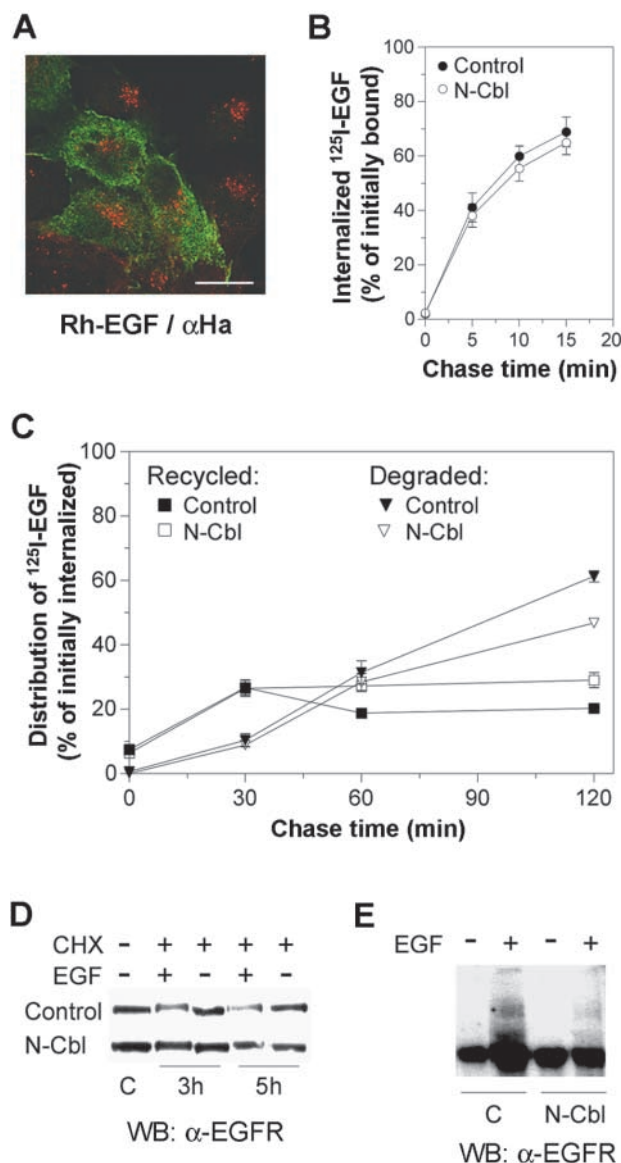


Figure 5. Overexpression of N-Cbl does not affect the endocytosis of EGF, but inhibits degradation of EGF and EGFR. (A) Rh-EGF (10 nM) was added to COS-1 cells transfected with a plasmid encoding HA-tagged N-Cbl. The cells were incubated for 15 min at 37°C, before being fixed and immunostained with anti-HA antibody. The HA-N-Cbl-positive cells demonstrate green fluorescence. Both transfected and nontransfected cells clearly endocytosed Rh-EGF (red fluorescence). Bar, 25 μm . (B) Mock-transfected COS-1 cells (transfected with empty pCDNA3) or COS-1 cells transfected with a plasmid encoding HA-N-Cbl were incubated with ^{125}I -EGF (0.2 nM) on ice for 15 min and chased at 37°C for the indicated time periods. Analysis of internalized EGF (●, ○) was performed as described (Skarpen et al., 1998). The data represent the mean of three independent experiments \pm SEM. (C) Mock-transfected COS-1 cells (■, ▼) or COS-1 cells transfected with a plasmid encoding HA-N-Cbl (□, ▽) were loaded with 8.5 nM ^{125}I -EGF for 20 min at 37°C. The cells were washed and stripped of surface-associated ^{125}I -EGF before being chased for indicated times. Analysis of recycled EGF (■, □), intact intracellular EGF and degraded EGF (▼, ▽) was performed as described in Materials and methods. The fraction representing intact intracellular EGF was not affected by N-Cbl and is not demonstrated. The data represent the mean of three independent experiments \pm SEM. (D) The effect of overexpression of N-Cbl on degradation of EGFR was investigated by Western blotting. Mock-

transfected COS-1 cells (control) and COS-1 cells transfected with a plasmid encoding HA-N-Cbl were incubated without or with EGF (10 nM) in the presence of CHX for 3 or 5 h. The cell lysates were subjected to SDS-PAGE and immunoblotting with anti-EGFR antibody. (E) The effect of overexpression of N-Cbl on ubiquitination of EGFR was investigated by immunoprecipitation and Western blotting. EGF (10 nM) was added to mock-transfected COS-1 cells (control) and to N-Cbl-transfected COS-1 cells, and EGF was bound for 15 min on ice. Then the cells were washed, and subsequently incubated at 37°C for 10 min. The cells were lysed and the lysate immunoprecipitated with antibody to conjugated ubiquitin. Immunoprecipitated material was subsequently analyzed by SDS-PAGE and immunoblotting with anti-EGFR antibody.

NH₄Cl, MG132 and lactacystin inhibit EGF-mediated degradation of the EGFR

The differential effect of EGF and TGF α with respect to degradation of the EGFR cannot be explained by differences in initial ubiquitination, nor by differences in endocytosis. However, only EGF caused a sustained ubiquitination, probably induced upon endocytosis. Degradation of the liganded EGFR is known to occur in lysosomes (Carpenter and Cohen, 1976; King et al., 1980a, 1980b). However, in analogy with other ubiquitinated receptors, proteasomes could additionally be involved in the degradation of the EGFR. To study this possibility, we investigated the effect of NH₄Cl, which counteracts the acidification of endocytic vesicles, and of the proteasomal inhibitors MG132 and lactacystin, on EGFR degradation. Hep2 cells were incubated with EGF (10 nM) in the presence of CHX and in the presence or absence of NH₄Cl, MG132 or lactacystin. As demonstrated in Fig. 6 A, NH₄Cl, MG132, and lactacystin all inhibited degradation of the EGFR, and MG132 was the most potent inhibitor of degradation. The observed EGFR degradation was the same when an antibody raised against the extracellular part of the EGFR was used (unpublished data). The data strongly suggest that lysosomal degradation of EGFR depends on proteasomal activity. We also investigated the effect of the same inhibitors with respect to EGFR activation by immunoblotting with an antibody to pY1173. As demonstrated in Fig. 6 B, phosphorylation of EGFR was sustained in the presence of NH₄Cl, MG132 and lactacystin, and MG132 affected the phosphorylation most efficiently. The sustained phosphorylation most likely reflects inhibited degradation of the phosphorylated receptor.

To exclude the possibility that the inhibition of EGFR degradation was a result of inhibited endocytosis, internalization of 0.2 nM ^{125}I -EGF was investigated in the presence or absence of NH₄Cl, MG132, or lactacystin. Hep2 cells

transfected COS-1 cells (control) and COS-1 cells transfected with a plasmid encoding HA-N-Cbl were incubated without or with EGF (10 nM) in the presence of CHX for 3 or 5 h. The cell lysates were subjected to SDS-PAGE and immunoblotting with anti-EGFR antibody. (E) The effect of overexpression of N-Cbl on ubiquitination of EGFR was investigated by immunoprecipitation and Western blotting. EGF (10 nM) was added to mock-transfected COS-1 cells (control) and to N-Cbl-transfected COS-1 cells, and EGF was bound for 15 min on ice. Then the cells were washed, and subsequently incubated at 37°C for 10 min. The cells were lysed and the lysate immunoprecipitated with antibody to conjugated ubiquitin. Immunoprecipitated material was subsequently analyzed by SDS-PAGE and immunoblotting with anti-EGFR antibody.

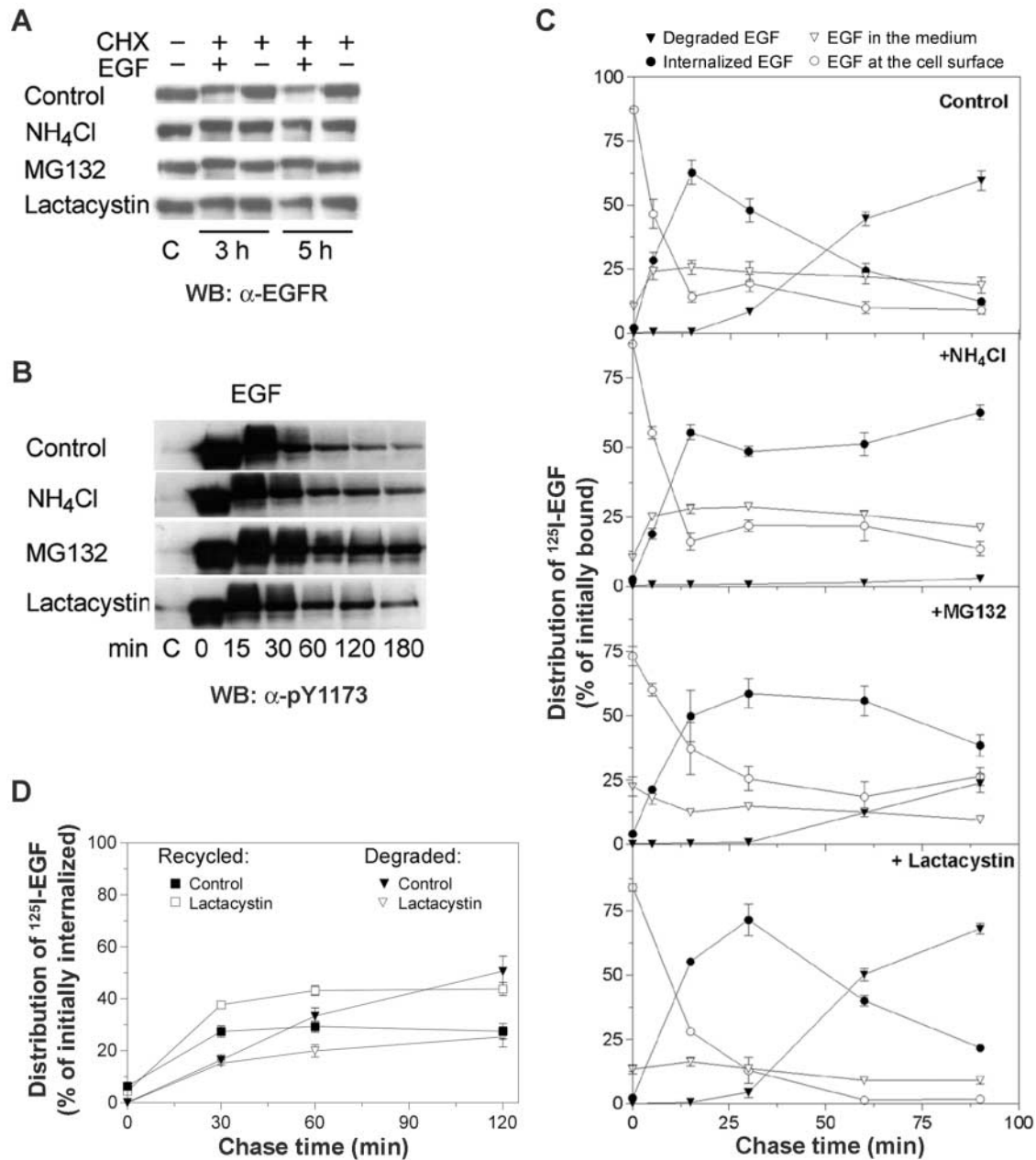


Figure 6. Degradation of the EGFR and ¹²⁵I-EGF in the presence of NH₄Cl, MG132, and lactacystin. (A–C) Hep2 cells were preincubated with either NH₄Cl, MG132 or lactacystin, as described in Materials and methods. (A) EGF (10 nM) and CHX (25 μ g/ml) was added, and the cells were further incubated at 37°C for the indicated time periods. The cells were lysed and subjected to SDS-PAGE and immunoblotting with antibody to EGFR. c, control cells not incubated with ligand or CHX. Both NH₄Cl, MG132 and lactacystin inhibited the degradation of EGFR. The presented data is representative of seven independent experiments. (B) EGF (10 nM) was added to cells on ice for 15 min before chase at 37°C for the indicated times in medium containing the same inhibitors as during the preincubation. The cells were lysed and subjected to SDS-PAGE and immunoblotting with antibody to activated EGFR (pY1173). c, control cells not incubated with ligand. (C) Cells preincubated with or without inhibitors were incubated with ¹²⁵I-EGF (0.2 nM) on ice for 15 min and chased in medium containing the inhibitors at 37°C for the indicated time periods. Analysis of EGF in the medium (∇), EGF at the cell surface (\circ), internalized EGF (\bullet) and degraded EGF (\blacktriangledown) was performed as described (Skarpen et al., 1998). Both NH₄Cl and MG132, but not lactacystin, inhibited the degradation of ¹²⁵I-EGF. (D) Hep2 cells were preincubated for 2 h with and without lactacystin. The cells were then loaded with 8.5 nM ¹²⁵I-EGF for 20 min at 37°C, and recycling and degradation was investigated as described in Fig. 5 C. Lactacystin was found to enhance recycling and to inhibit degradation of ¹²⁵I-EGF. The fraction representing intact intracellular EGF was not affected by lactacystin and is not demonstrated. The data in C and D represent the mean of 3 independent experiments \pm SEM.

were preincubated with NH₄Cl, MG132 or lactacystin at 37°C before incubation with ¹²⁵I-EGF on ice for 15 min. The cells were subsequently washed and incubated at 37°C with medium containing the inhibitors. As shown in Fig. 6

C, neither NH₄Cl, MG132, nor lactacystin, had any effect on the internalization of ¹²⁵I-EGF. Both NH₄Cl and MG132, but not lactacystin, inhibited degradation of ¹²⁵I-EGF at the low concentration of ligand used in this experi-

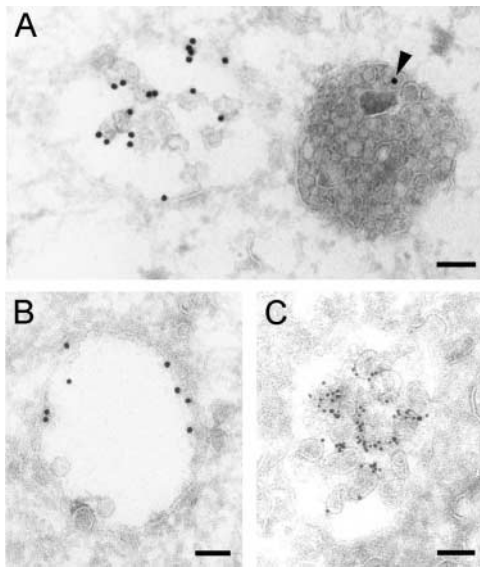


Figure 7. Translocation of EGF-EGFR to internal membranes of MVBs depends on proteasomal activity. Control and lactacystin-treated Hep2 cells were incubated with EGF for 15 min on ice followed by chase in EGF free medium at 37°C for 1 h. To localize endocytosed EGF, ultra-thin frozen sections were labeled with anti-EGF antibodies followed by 15 nm protein A gold. In control cells (A) EGF is localized within MVBs with a high number of internal vesicles, and small amounts of labeling is also found within electron dense, compact MVBs (arrowhead). In lactacystin-treated cells (B) EGF is localized to MVBs with very few internal vesicles. (C) Lactacystin-treated Hep2 cells (preincubated for 2 h) were incubated with 10 nm BSA-gold for 60 min at 37°C. Endocytosed gold was found to localize within MVBs with high numbers of internal vesicles. Bars, 100 nm.

ment. To study effects of lactacystin on recycling and degradation of EGFR-bound EGF, 8.5 nM ^{125}I -EGF was used, and the analysis was performed essentially as described (Babst et al., 2000). As demonstrated in Fig. 6 D, lactacystin slightly enhanced recycling and inhibited degradation of ^{125}I -EGF. The fact that lactacystin inhibits degradation of EGFR-bound EGF at high ligand concentration suggests that proteasomal activity is involved in efficient delivery to an environment where EGF is degraded.

The finding that MG132, but not lactacystin, inhibits degradation of very low concentrations of EGF can be explained by the possibility that MG132, which is a peptide aldehyde, does not specifically inhibit proteasomal proteases. We used a Cathepsin B enzyme assay to study the potential effect of MG132 and lactacystin on Cathepsin B, reported to be important for degradation of EGF and EGFR (Authier et al., 1999). We found that MG132 caused an 80% inhibition of Cathepsin B, whereas lactacystin only slightly (<10%) inhibited Cathepsin B. Therefore, the strong inhibitory effect of MG132 on degradation of EGF can be explained by the inhibitory effect of MG132 on Cathepsin B.

The effect of lactacystin on ligand-induced trafficking of the EGFR was further studied by immuno-EM. Control cells or cells pretreated with lactacystin or MG132 were incubated with EGF (10 nM) for 15 min on ice, followed by chase in ligand-free, prewarmed medium with inhibitor. After 1 h chase, both EGF and the EGFR (unpublished data)

localized to MVBs. However, both the appearance of MVBs and the distribution of EGF and EGFR inside MVBs were different in control cells compared with in cells treated with lactacystin and MG132. In control cells the labeling for EGF was found in MVBs with a high number of internal vesicles, and >80% of the labeling localized to these internal membranes (Fig. 7 A). In cells treated with lactacystin or MG132 (Fig. 7 B; unpublished data), labeling for EGF was mainly found on MVBs with few internal vesicles, and >60% of the labeling was on the outer limiting membrane. This shows that proteasomal activity is involved in the translocation of EGFR from the limiting membrane to internal membranes within MVBs.

To investigate whether proteasomal inhibitors affected all intracellular sorting, we studied the fluid phase endocytosis of BSA-coated colloidal gold. As demonstrated in Fig. 7 C, BSA-gold was transported efficiently to MVBs filled with internal membranes, demonstrating that lactacystin did not affect formation of MVBs in general.

To characterize the endosomes to which EGF and EGFR localize in the presence of lactacystin more closely, we added Rh-EGF and FITC-transferrin (Tf) to cells in the presence or absence of lactacystin. Upon incubation for 15 min, most EGF colocalized with Tf. Upon incubation for 60 min, there was no significantly increased colocalization of EGF and Tf in lactacystin-treated cells, compared with control cells (unpublished data). Therefore, the endosomes depicted in Fig. 7 B do not appear to be part of the recycling compartment.

Proteasomal inhibitors do not inhibit degradation of EGF-EGFR by depleting cellular ubiquitin

A side effect of incubating cells with proteasomal inhibitors could be depletion of intracellular ubiquitin (Swaminathan et al., 1999). Therefore, we investigated the effect on EGFR ubiquitination of incubating Hep2 cells with lactacystin for 3 and 5 h. Hep2 cells were preincubated with both lactacystin and CHX for 3 and 5 h (the same way degradation of EGFR was studied). As shown in Fig. 8, the EGFR was ubiquitinated equally efficiently by addition of EGF regardless of preincubation with lactacystin. This demonstrates

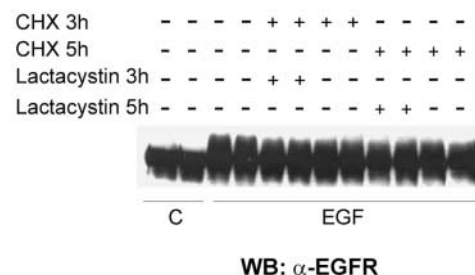


Figure 8. Preincubation of Hep2 cells with lactacystin for 3 and 5 h does not inhibit subsequent EGF-induced ubiquitination of the EGFR. Hep2 cells were preincubated with or without lactacystin and CHX for 3 or 5 h. Then control cells and cells pretreated with lactacystin/CHX were incubated without or with EGF for 15 min on ice before being washed and incubated at 37°C for 10 min. The cell lysates were subjected to SDS-PAGE and immunoblotting with antibody to EGFR. The upsmeared illustrates ubiquitination.

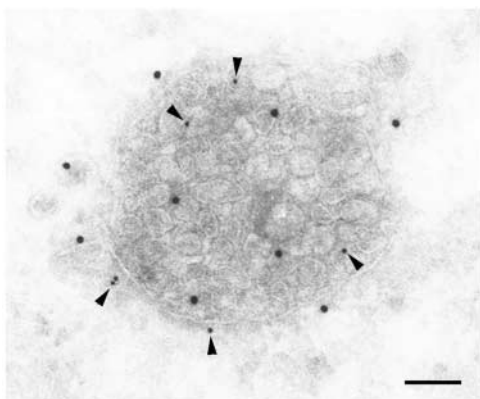


Figure 9. The COOH-terminal part of EGFR-GFP is intact upon translocation of EGFR to inner membranes of MVBs. Hep2 cells transfected with EGFR-GFP, as described in Materials and methods, were incubated with 10 nM EGF for 30 min at 37°C before processing for immuno-EM. Thawed cryosections were double labeled using anti-GFP antibodies followed by 15 nm protein A gold and anti-EGFR antibodies followed by 10 nm protein A gold (arrowheads). The results show that both GFP- and EGFR-labeling localize to the limiting membrane as well as to inner membranes of MVBs. Bar, 100 nm.

that reduction of intracellular ubiquitin resulting from preincubation of cells with lactacystin was not sufficient to inhibit EGF-induced polyubiquitination of the EGFR and strongly supports the notion that proteasomal activity is essential for efficient transport of EGF-bound EGFR to inner membranes of MVBs.

The EGFR is not a direct target of proteasomal proteases

An important consideration is the nature of the proteasomal substrate. To clarify whether the EGFR is itself cleaved during sorting to MVBs, we transfected Hep2 cells with a plasmid encoding the EGFR-green fluorescent protein (GFP) fusion protein. This fusion protein is endocytosed and degraded exactly like wild-type EGFR upon binding EGF (Carter and Sorkin, 1998). Therefore, we investigated by immuno-EM whether or not GFP could be detected on inner membranes of MVBs. As illustrated in Fig. 9, we found that upon incubation of the cells with EGF for 30 min at 37°C, labeling for the EGFR as well as for GFP could be detected both on the limiting and internal membranes of MVBs. Quantification showed that ~60% of anti-EGFR labeling and 50% of the anti-GFP labeling localized to the internal membranes. As the anti-EGFR antibody recognizes the cytosolic part of the EGFR and GFP is a COOH-terminal extension of the EGFR, the experiment demonstrates that the cytosolic part of the EGFR is intact even when the EGFR localizes to internal membranes of MVBs. This shows that although proteasomal activity is needed for efficient translocation of EGFR to internal membranes of MVBs, the EGFR itself is not a direct proteasomal target.

Discussion

In the present work we have expanded on our previous observation that the EGFR was polyubiquitinated at the plasma membrane (Stang et al., 2000). We have studied

EGFR ubiquitination using Hep2 cells, and we now report that the EGFR becomes polyubiquitinated even when cells are incubated with ligand on ice. As endocytosis of the EGFR does not happen when cells are incubated on ice, our results confirm the finding that EGFR is ubiquitinated at the plasma membrane before endocytosis. Consistently, we observed tyrosine phosphorylation of c-Cbl and recruitment of c-Cbl to the plasma membrane upon activation of the EGFR on ice. A possible scenario is that c-Cbl-dependent ubiquitination is important for ligand-induced endocytosis of the EGFR. This would be in agreement with the conclusion that c-Cbl is required for endocytosis of the colony stimulating factor-1 receptor (Wang et al., 1996, 1999; Lee et al., 1999). However, our present results demonstrate that overexpression of N-Cbl, which acts dominant negatively with respect to ubiquitination of the EGFR, does not affect EGFR endocytosis. This is in accordance with previously published views (Levkowitz et al., 1998; Thien et al., 2001).

We have taken advantage of the two different EGFR ligands, EGF and TGF α , to induce different trafficking of the EGFR upon endocytosis. Interestingly, both ligands initially induced ubiquitination and endocytosis of the EGFR to the same extent, even though TGF α very inefficiently induced transport of the EGFR to internal vesicles of MVBs and lysosomal degradation of the EGFR. This clearly illustrates that the initial ubiquitination induced by activation of the EGFR is not a signal for lysosomal sorting. It should be noted that the ubiquitination induced by EGF lasted longer than the ubiquitination induced by TGF α . The sustained ubiquitination induced by EGF correlated with the sustained tyrosine phosphorylation of the EGFR and c-Cbl, and therefore the sustained kinase activity of the EGFR. Because EGFR kinase activity has been demonstrated to be required for transport of the EGFR from the limiting membrane to the internal vesicles of MVBs (Felder et al., 1990), the sustained ubiquitination seen upon incubation with EGF could be important for trafficking of EGFR to lysosomes. Interestingly, Katzmann et al. (2001) recently reported ubiquitin-dependent sorting of the vacuolar hydrolyase carboxypeptidase S to internal membranes of MVBs in yeast. They further characterized a conserved endosomal sorting complex containing the protein Vps23. This protein contains a ubiquitin conjugating-like domain (Babst et al., 2000). The mammalian homologue of Vps23 is the protein encoded by the tumor susceptibility gene 101 (Tsg101), and this protein has been demonstrated to function in late endosomal trafficking. In fact, in *tsg 101* mutant cells, endocytosed EGFR was rapidly recycled back to the cell surface and inefficiently degraded (Babst et al., 2000). Katzmann et al. proposes a model whereby ubiquitinated proteins bind to the sorting complex containing Vps23. Several other class E Vps proteins are suggested to be important for the subsequent sorting, as well as for recruitment of deubiquitinating enzymes, like Doa4 (Katzmann et al., 2001).

The importance of proteasomal activity in lysosomal trafficking of EGFR is supported by our finding that lactacystin inhibits degradation of the EGFR as efficiently as does NH₄Cl. Our present immuno-EM data show that inhibition of proteasomal activity with lactacystin causes retention of EGFR in MVBs at an early stage of formation. Consistently,

we found, as did Levkowitz et al. (1998), that inhibition of proteasomes promoted recycling and inhibited degradation of EGF. However, the effect of lactacystin on recycling and degradation of EGF was smaller than the effect observed in *tsg 101* mutant cells (Babst et al., 2000) and smaller than the effect observed upon overexpression of N-Cbl (transfection efficiency considered). That degradation of EGF is inhibited more efficiently by overexpression of N-Cbl and in *tsg 101* mutant cells compared with in cells incubated with lactacystin, suggests the involvement of proteasomes downstream of ubiquitinated EGFR interacting with a complex containing Tsg 101. Our data show that proteasomal activity is not required for the formation of MVBs in general, as lactacystin does not inhibit transport of BSA-gold to MVBs. Morphologically the EGF/EGFR positive compartments found upon inhibition of proteasomal activity resembled the EGFR positive compartments found upon incubation with TGF α . We found no significantly increased colocalization of EGF and Tf upon incubating cells with lactacystin.

Although ubiquitinated EGFR can be recognized and degraded by purified 26S proteasomes in vitro (Levkowitz et al., 1999), our results show that the cytosolic part of the EGFR is intact on internal membranes of MVBs, and therefore demonstrate that the EGFR itself is not a direct proteasomal target in vivo. It was earlier also demonstrated that although the ubiquitination machinery and proteasomal activity is needed for degradation of the growth hormone receptor, neither the cytosolic tail of the growth hormone receptor is a proteasomal target (van Kerkhof et al., 2001). Therefore, the nature of the proteasomal target(s) is still unclear.

In conclusion, our data demonstrate that there is an initial ubiquitination of the EGFR at the plasma membrane, but that this ubiquitination per se does not encode EGFR degradation and that initiation of endocytosis of the EGFR does not depend on ubiquitination. Upon conditions where the EGFR kinase activity is secured, the ubiquitination of the EGFR is sustained. Eventually, activated EGFR is efficiently transported to internal vesicles of MVBs in a proteasome dependent process. Therefore, the sustained ubiquitination of the EGFR seems to be a way of engaging ubiquitin-interacting proteins, and proteasomal cleavage eventually facilitates the transport of the EGFR to a highly degrading environment.

Materials and methods

Materials

Human recombinant EGF was from Bachem Feinchemikalien AG, human recombinant TGF α was from R&D Systems, Inc., 125 I-EGF was from Amersham Pharmacia Biotech, MG132 from Affinity Research Products Ltd., and PD153035 was from Tocris Cookson Ltd. EZ-link sulfo-NHS-LC-Biotin was from Perbio Science Ltd., alkaline phosphatase-conjugated streptavidine from Dako Corporation, and rhodamine-EGF (Rh-EGF) and FITC-Tf were from Molecular Probes, Inc. BSA-gold was prepared according to Slot and Geuze (Slot and Geuze, 1985). Fugene was from Roche Diagnostics Corporation. Other chemicals were from Sigma-Aldrich unless otherwise noted.

Cell culture and treatment

Hep2 and COS-1 cells were grown in DME (BioWhittaker) containing penicillin-streptomycin-fungizone mixture (BioWhittaker), L-glutamine (2 mM) (BioWhittaker), and FBS (BioWhittaker) (5% vol/vol in the case of Hep2 cells and 10% for COS-1 cells). The cells were plated at a density of 15,000 cells/cm 2 48 h before experiments. In pulse-chase experiments, cells were incubated with ligand in MEM without bicarbonate with 0.1% BSA on ice for 15 min, followed by washing three times in ice-cold PBS to

remove unbound ligand, and subsequent chase in ligand-free MEM without bicarbonate at 37°C.

Antibodies

Sheep anti-EGFR was from Gibco Life Technologies, and Fitzgerald Industries International, Inc., rabbit anti-EGFR, rabbit anti-EGF, and rabbit anti-c-Cbl from Santa Cruz Biotechnology, Inc., mouse inactivated EGFR (pY1173) and mouse anti-phosphotyrosine from Upstate Biotechnology, rabbit anti-conjugated ubiquitin from Sigma-Aldrich, mouse anti-hemagglutinin (HA) from Roche Diagnostics, rabbit anti-GFP from AbCam Ltd., rabbit anti-mouse IgG from Cappel, ICN Biomedicals. Peroxidase-conjugated donkey anti-sheep, peroxidase-conjugated donkey anti-mouse, Cy 2 -conjugated donkey anti-sheep and rabbit anti-sheep IgG were all from Jackson ImmunoResearch Laboratories. Alexa Fluor 594-conjugated goat anti-rabbit was from Molecular Probes.

Immunoprecipitation

Cells were lysed in immunoprecipitation buffer, as described (Stang et al., 2000). Protein A or protein G-coupled Sepharose beads (Amersham Pharmacia Biotech) were incubated with antibody for 1 h at room temperature and subsequently washed twice with immunoprecipitation buffer, before the cell lysates were added. Immunoprecipitation was performed at 4°C for 1 h. Immunoprecipitation with antibody to conjugated ubiquitin and SDS-PAGE and immunoblotting was performed as described (Stang et al., 2000).

Biotinylation of surface-localized EGFR

Biotinylation was performed at 4°C, essentially as described (Corbeil et al., 1999), using 2 mM biotin. The EGFR was immunoprecipitated using rabbit anti-EGFR and protein A-coupled Sepharose beads (Amersham Pharmacia Biotech). The immunoprecipitate was subjected to SDS-PAGE and electrotransferred to PVDF transfer membrane (Hybond-P; Amersham Pharmacia Biotech). The membrane was incubated with alkaline phosphatase-conjugated streptavidin or with sheep anti-EGFR antibody and alkaline phosphatase-conjugated anti-sheep antibody. Reactive bands were analyzed by enhanced chemifluorescence (Amersham Pharmacia Biotech). The band intensity was measured using a phosphorfluorimager (Molecular Imager FX; Bio-Rad Laboratories).

Plasmids and transfection of cells

The plasmid EGFR-GFP, encoding a fusion protein of EGFR and enhanced GFP (Carter and Sorokin, 1998) was provided by Dr. Alexander Sorokin. Hep2 cells were transfected with this plasmid using Fugene 24 h upon plating. HA-tagged c-Cbl in the pJZenNeo vector (Andoniou et al., 1994) was provided by Dr. Robin M. Scaife. By polymerase chain reaction a 1,121-bp fragment, corresponding to the HA tag and the 357 amino acids constituting N-Cbl was subcloned into pCDNA3. This plasmid was transfected into COS-1 cells using Fugene 24 h after plating. Transfected cells were analyzed 48 h after transfection.

Immunocytochemistry and confocal microscopy

Cells were plated on 12-mm coverslips (MENZEL-GLÄSER®). After experiments, cells were washed once in cytoskeleton buffer (NaCl [137 mM], KCl [5 mM], Na $_2$ HPO $_4$ [1.1 mM], KH $_2$ PO $_4$ [0.4 mM], glucose [5.5 mM], NaHCO $_3$ [4 mM], MES [10 mM], EGTA [2 mM], MgCl $_2$ [2 mM]) and subsequently fixed with paraformaldehyde (4% wt/vol) (Riedel-de Haën AG) in Soerensen's phosphate buffer for 20 min on ice. Cells were washed three times with cytoskeleton buffer before permeabilization with Triton X-100 (0.1% wt/vol in cytoskeleton buffer) for 10 min. The fixed and permeabilized cells were preincubated with BSA (1% wt/vol in PBS) for 30 min before incubation with primary antibodies for 1 h. The coverslips were washed with PBS and incubated with secondary antibodies for 30 min before mounting with Dako fluorescent mounting medium with Na $_2$ S $_2$ O $_8$ (15 mM). The cells were examined using a confocal microscope (Leica TCS).

125 I-EGF interaction experiments

The cells were incubated as described in legends to Figs. 5 and 6. Internalization and degradation of EGF was analyzed as previously described (Skarpen et al., 1998). Additionally, recycling of EGF was analyzed essentially as described (Babst et al., 2000), loading cells with 8.5 nM 125 I-EGF in MEM without bicarbonate and with 0.1% BSA. Upon loading, the surface-localized radioactivity was removed by a glycine-buffered solution, pH 3.0 (Babst et al., 2000). Upon chase at 37°C, the medium was analyzed for degraded and recycled EGF as described (Skarpen et al., 1998), and the cells were analyzed for recycled EGF (released by the pH 3.0 buffer) and for internalized EGF (cells solubilized by 1 M NaOH after treatment with

low pH). The cpm representing recycled EGF in the medium and at the cell surface were combined in one fraction.

Inhibition of lysosomal or proteasomal activity

Hep2 cells were preincubated with either NH_4Cl (10 mM) for 10 min, MG132 (10 μM) or lactacystin (50 μM) for 1 or 2 h at 37°C. To measure degradation of the EGFR, EGF (10 nM) and CHX (25 $\mu\text{g}/\text{ml}$) were added and the cells incubated at 37°C for the indicated times. Cells were lysed and subjected to SDS-PAGE and immunoblotting with antibody to EGFR. For immuno-EM, cells preincubated with the different inhibitors were incubated with EGF (10 nM) on ice for 15 min followed by chase at 37°C for the indicated time periods in the presence of inhibitors.

Cathepsin B enzyme assay

The activity of Cathepsin B (Calbiochem) in the presence of lactacystin and MG132 was analyzed by use of Colorimetric Substrate I (Calbiochem) according to the manufacturer's instructions. The enzyme assay was performed three times with three parallels for each condition.

Immuno-EM

The cells were incubated as described in legends to figures. The cells were subsequently washed with PBS, fixed with paraformaldehyde (4% wt/vol) and glutaraldehyde (0.1% wt/vol) in Sorensen's phosphate buffer and processed for cryosectioning and immunolabeling (Griffiths et al., 1984). Bound antibodies were visualized using protein A gold, a gift of Dr. G. Posthuma. When the primary antibody was mouse or sheep IgG, incubation with rabbit anti-mouse or rabbit anti-sheep IgG was used as an intermediate step between the primary antibody and protein A gold. The sections were examined using a Philips CM 120 electron microscope. To quantify labeling of MVBs, the total number of gold particles found on MVBs (100%) were divided into two groups, gold particles associated with the outer limiting membrane and gold particles associated with internal membranes, and the percentage distribution of the labeling was calculated. In each experiment a minimum of 100 gold particles were counted on MVBs.

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