Ligand-induced Lysosomal Epidermal Growth Factor Receptor (EGFR) Degradation Is Preceded by Proteasome-dependent EGFR De-ubiquitination*

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Studies on the differential routing of internalized epidermal growth factor receptors (EGFRs) induced by EGF, TGFα, and the superagonist EGF-TGFα chimera E4T suggested a correlation between receptor recycling and their mitogenic potency. EGFR sorting to lysosomes depends on its kinase domain and its ubiquitination by Cbl proteins. Proteasomes have also been proposed to regulate EGFR degradation, but the underlying mechanism remains obscure. Here we evaluated EGFR activation, Cbl recruitment, EGFR ubiquitination and degradation in response to EGF, TGFα, and E4T. We also determined the fate of activated EGFRs and Cbl proteins by using v-ATPase (bafilomycin A1) and proteasome (lactacystin) inhibitors. Our results demonstrate that E4T and TGFα provoke decreased Cbl recruitment, EGFR ubiquitination and EGFR degradation compared with EGF. Furthermore, bafilomycin treatment blocks EGFR but not c-Cbl degradation. In contrast, lactacystin treatment blocks EGFR-induced c-Cbl degradation but does not block EGFR degradation, even though lactacystin causes a minor delay in EGFR degradation. Strikingly, even though bafilomycin completely blocks EGFR degradation, it does not prevent EGFR de-ubiquitination upon prolonged EGF stimulation. Strikingly, when combined with bafilomycin, lactacystin treatment stabilizes the ubiquitinated EGFR and prevents its de-ubiquitination. We conclude that the enhanced EGFR recycling that has been observed in HER-14 cells following TGFα or E4T stimulation correlates with decreased EGFR ubiquitination and EGFR degradation, and that proteasomal activity is required for de-ubiquitination of the EGFR prior to its lysosomal degradation.

Polypeptide growth factors play an important role in the regulation of cell division. In various types of epithelial carcinomas the overexpression of human transforming growth factor α (TGFα) is frequently observed. This is often accompanied by overexpression of ErbB receptors (ErbB1 also called the EGFR, ErbB2) and has been associated with poor prognosis and poor response to chemotherapy (1, 2).

EGF and TGFα are highly homologous molecules; both are members of the epidermal growth factor family (3) and exert their biological function through binding to the EGFR, a member of the ErbB receptor family. Ligand-induced receptor dimerization is a prerequisite for receptor activation, which triggers the intrinsic kinase activity resulting in the phosphorylation of several tyrosine residues at the C-terminal region of the receptor (4). The phosphorylated tyrosine residues serve as docking sites for several SH2 domain-containing proteins thereby conveying the biological signal from outside to the inner side of the cell. Multiple protein-protein interaction cascades follow, resulting in the activation of MAPK and eventually in cell division. Ligand binding to the EGFR also triggers the internalization and subsequent degradation of the activated receptor (4). This process leads to clearing of activated receptors from the cell surface thereby desensitizing the cells for mitogenic signals (5–7). Recruitment of active receptors to clathrin-coated pits forms the first step in the internalization process. Clathrin-coated pits mature into clathrin-coated vesicles, which subsequently fuse with the early endosome. From the early endosome, free EGFRs recycle back to the cell surface, whereas ligand-bound receptors undergo receptor kinase-dependent sorting into the internal vesicles of the multivesicular bodies (MVBs) (8). The maturation of early endosome to late endosome is accompanied by accumulation of EGF-EGFR complexes in the internal vesicles of MVBs (9) resulting in sequestration of the cytoplasmic domain, thereby preventing it from interacting with cytoplasmic target molecules. Subsequently, the late endosome fuses with pre-existing lysosomes leading to lysosomal degradation of ligand-receptor complexes by the lysosomal proteases (10). The pH of the lumen of the endocytic compartments decreases along the endocytic route due to the action of the vacuolar ATPase. The EGF-EGFR complex is resistant to the mildly acidic pH of early and late endosomes and remains intact along the endocytic route (5). Upon reaching the lysosome, both ligand and receptor are degraded by the lysosomal proteases (9, 10).

Ligand-induced polyubiquitination of the EGFR (11) is an essential factor in the down-regulation of activated EGFRs (12–16). Ubiquitination of cargo proteins requires the ATP-dependent activation and covalent binding of the 76 amino acids ubiquitin molecule by the ubiquitin-activating enzyme (designated E1), after which ubiquitin is transferred to an E2 ubiquitin-conjugating enzyme. E2 enzymes ubiquitinate target substrate proteins in concert with either E3 ubiquitin ligase enzymes or with E3 RING finger adapter proteins. This results in the covalent attachment of ubiquitin molecules to a lysine residue on a substrate protein. This process is often iterated resulting in multiple attachments of ubiquitin molecules to either a different lysine residue on a substrate protein or to a
lysine residue on previously attached ubiquitin (17).

Ubiquitination of the EGFR is critically dependent on the activity of Cbl adapter proteins (18, 19). Cbl-b and c-Cbl are members of the Cbl adapter family that constitute an important part of the ubiquitination machinery. Both undergo tyrosine phosphorylation in response to EGFR activation (20, 21). Negative regulation of activated EGFRs requires an intact SH2 variant N-terminal region (19), through which Cbl interacts with phosphotyrosine 1045 in the C-terminal tail of the EGFR (13) and intact linker and RING finger domains to which ubiquitin-conjugating enzymes (E2s) bind (22, 23). This dual binding property of Cbl proteins facilitates recruitment of the ubiquitination machinery to activated EGFRs, an event that results in polyubiquitination of the EGFRs. Interestingly, Levkowitz et al. (12) demonstrated that oncogenic Cbl proteins inhibit down-regulation and enhance recycling of internalized EGFRs.

In general, polyubiquitin serves as a signal for degradation by the 26S proteasome. Thus, even though degradation of activated EGFRs can be mediated by lysosomal proteases (5, 10, 24), it has been reported that proteasomes also play an important role in the ligand-induced degradation of ubiquitinated EGFRs. Indeed, blocking proteasomal activity with the non-proteasome-specific peptide aldehyde MG132 resulted in accumulation of full length EGFR and prevention of receptor degradation (16, 25). Therefore, the exact mechanism responsible for the degradation of the EGFR remains obscure and in particular the role of the proteasome in the degradation process of the EGFR is an issue of debate.

Multiple EGFR ligands are known to differ in their mitogenic activity. For example in various model systems, TGFβ is a more potent mitogen than EGF (26–29). Moreover, several chimeric EGF-TGFβ growth factors that were generated in our department, show superagonist activity in both 32D1 and HER-14 model systems and are able to induce similar mitogenic responses as EGF but at 10-fold lower concentrations (29, 30). One such superagonist ligand is E4T, in which EGF sequences after the fourth cysteine of the EGF module have been replaced by the corresponding residues of TGFβ. In addition, similar to TGFβ, EGFR binding capacity of these superagonists was found to be more sensitive to low pH compared with EGF (30). Consequently, TGFβ and the superagonists might dissociate earlier in the extracellular system than EGF leading to enhanced recycling of the EGFR. Indeed, while EGFR internalization kinetics are similar in response to EGF, TGFβ, or E4T (29, 30), recycling assays suggested a correlation between superagonist-induced receptor recycling and enhanced mitogenic activity in both HER-14 (30) and 32D1 (29) model systems. In addition, superagonist ligands, including E4T, show enhanced on/off rates as measured by Biacore experiments (31) as well as decreased ligand depletion from the medium (29).

To study the enhanced receptor recycling induced by TGFβ and EGF/TGFβ chimeric superagonist ligands we hypothesized that Cbl proteins are inefficiently “coupled” to these growth factors. In the present study the functional coupling of Cbl adapter proteins to EGF, TGFβ and superagonist ligand E4T was investigated. The degradation process of the EGFR was further analyzed by studying the differential effects of lysosomal and proteasomal inhibitors on the degradation of the EGFR. We have found that the lysosomal degradation of the EGFR is preceded by its de-ubiquitination.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—NIH 3T3 cells stably transfected with human EGFR (HER-14) (32) were used throughout this study. Cells were cultured on flasks coated with 0.1% gelatin and grown at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% newborn calf serum. Cells were trypsinized when confluent and seeded in flasks for regular maintenance.

**Antibodies and Materials**—Anti-c-Cbl (clone R2) was a generous gift from W. Langdon. Anti-Cbl (clone C15), anti-Cbl-b (clone H9262), and anti-EGFR antibodies (mAb 528 and polyclonal antibody 1005) were obtained from Santa Cruz Biotechnology. Anti-phosphotyrosine (4G10) and anti-EGFR mAb LA22 were obtained from Upstate Biotechnology, and anti-ubiquitin antibody from Sigma (pr. no. U5379). Anti-EGFR (clone13), as well as goat anti-rabbit (GARPO) and goat anti-mouse (GAMPO) antibodies linked to horseradish peroxidase were purchased from Signal Transduction Laboratories. Anti-EGFR antibody Ab12 was obtained from Neomarkers. Recombinant growth factors were produced and quantified as previously described (30). Sepharose beads coupled to protein A were obtained from Amersham Biosciences. Bafilomycin A1 and lactacytin were obtained from Calbiochem.

**Cell Stimulation and Pretreatment with Inhibitors**—Near confluent HER-14 cells were first washed twice with phosphate-buffered saline and incubated with serum-free medium for 24 h. Growth factors were then resuspended in DMEM containing 50 mm BES (N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid), pH 6.8. Then, growth factors were added to a final concentration of 100 ng/ml. Lactacytin and bafilomycin A1 were dissolved in MeSO and further diluted in serum-free DMEM (final concentration of lactacytin is 10 μM and bafilomycin is 0.25 μM) and added to cells one hour prior to stimulation.

**Cell Lysis**—Cells were washed twice with ice-cold PBS and adherent cells were lysed during a 20-min incubation at 4 °C in lysis buffer (150 mm NaCl, 25 mm Tris pH 7.5, 1% Brij97, 5 mm EDTA pH 8.0, 1 mm Na3VO4, 1 mm NaF, 1 mm phenethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin). This buffer contains inhibitors of de-ubiquitinating enzymes. Cell lysates were subsequently transferred to Eppendorf tubes and centrifuged for 10 min at 4 °C at 1100 × g in an Eppendorf centrifuge to remove nuclei and cell debris. Samples were prepared for SDS-PAGE analysis by adding Laemmli sample buffer to cleared whole cell lysates and then heated for 5 min at 95 °C prior to loading SDS-PAGE gels under reducing conditions.

**Immunoprecipitation, Recapture, and Immunoblotting**—Anti-c-Cbl clone C15 or anti-c-Cbl R2 antisera were used to immunoprecipitate c-Cbl protein. Anti-EGFR clone 528 was used throughout this study to immunoprecipitate EGFR. Antibodies used in the immunoprecipitation were first coupled to protein A-Sepharose beads for 30–60 min at 4 °C. Cleared cell lysates were then added to the antibody-absorbed beads and incubated for at least 2 h at 4 °C. Unbound cellular proteins were then removed by washing 3 times with ice-cold lysis buffer and once with ice-cold phosphate-buffered saline. Immunocomplexes were then re-suspended in 2× Laemmli sample buffer. Samples were heated for 5 min at 95 °C and kept at −20 °C or loaded directly onto 8% SDS-PAGE gels. Immunocomplexes separated by SDS-PAGE were transferred to nitrocellulose filters. Blots were first blocked with 5% bovine serum albumin, except for ubiquitin blots, which were blocked with 5% milk in TBST (Tris-buffered saline-Tween). After incubation with primary antibody, filters were washed in TBST, incubated with appropriate HRP-linked secondary antibodies, and washed again with TBST prior to visualization of proteins with enhanced chemiluminescence. For recapture experiments, primary anti-EGFR (clone 528) precipitates were split in two equal parts during the phosphate-buffered saline wash, of which one part was processed as detailed above. 50 μl of 1% SDS was added to the other part of the primary immunoprecipitate and subsequently incubated for 5 min at 95 °C to denature all proteins in the immunoprecipitate. After boiling, samples were allowed to cool to room temperature and 9 μl of recapture buffer was added to each half of the SDS, after which samples were centrifuged briefly. The resulting supernatants were transferred to beads coated with 2 μg of anti-EGFR recapture antibodies (LA22 or clone13) for 16 h at 4 °C. Samples were washed and prepared for SDS-PAGE as described above. Image analysis was performed using the NIH-Image software program available from rsb.info.nih.gov/nih-image. Briefly, signals of interest were overlaid by a defined rectangle and the mean pixel value (MPV) of these boxed areas was calculated. In addition, the background MPV was determined in areas not containing any specific signal. The background MPV value was then subtracted from experimental (including experimental control) MPV values. The resulting values were then normalized relative to the appropriate and similarly calculated control bands, or they were expressed as a percentage of time 0, and plotted as shown in the figures. All experiments shown are representative of at least two, and mostly three or more independent experiments (data not shown).
Inefficient EGFR Ubiquitination following Superagonist Stimulation

Although EGF, TGFα, and the superagonist E4T induce similar initial EGFR kinase activation and EGFR internalization, TGFα and E4T differ markedly from EGF in their mitogenic potency (29–31). Moreover, E4T requires 10-fold lower concentration to induce similar MAPK and mitogenic signaling as EGF and TGFα (29–31). However, the kinetics of EGFR activation in response to these growth factors has not been studied in detail. To investigate this issue, HER-14 cells were serum-starved and treated with saturating concentrations of EGF, TGFα, or E4T for various periods of time followed by evaluation of the tyrosine phosphorylation status of the EGFR. As seen in Fig. 1A (and data not shown), the tyrosine phosphorylation of the 180-kDa species of the EGFR is comparable for all growth factors, consistent with the use of saturating ligand concentrations which lead to similar cell surface receptor occupancy levels. In addition, Fig. 1A and B show that in response to EGF stimulation the anti-phosphotyrosine antibody immunoreacted with high molecular weight species of the EGFR, represented by the smear above the 180-kDa band.

The high molecular weight smear is markedly decreased in TGFα- and E4T-treated cells compared with EGF. This decrease is already visible after 5 min of EGFR activation (see Fig. 1B) and becomes more prominent after 15 min of receptor stimulation. It is well established that ligand-dependent EGFR activation induces receptor polyubiquitination (12), which leads to a variable increase in the molecular weight of the EGFR according to the number of ubiquitin molecules that are covalently attached to the receptor. This influences the electrophoretic mobility of ubiquitinated EGFRs on a SDS-PAGE gel and as a result ubiquitinated EGFRs appear as a smear above the 180-kDa position that corresponds with the non-ubiquitinated EGFR. To determine whether the phosphorylated high molecular weight smear corresponds with polyubiquitinated species of the EGFR, HER-14 cells were stimulated with EGF, TGFα, and E4T for the indicated period of time. The EGFR was immunoprecipitated and immunoblotted with anti-EGFR antibodies (upper panel) and, subsequently, reprobed with anti-EGFR (Ab12) antibodies (lower panel).

Results

Inefficient EGFR Ubiquitination following Superagonist Stimulation—Although EGF, TGFα, and the superagonist E4T induce similar initial EGFR kinase activation and EGFR internalization, TGFα and E4T differ markedly from EGF in their mitogenic potency (29–31). Moreover, E4T requires 10-fold lower concentration to induce similar MAPK and mitogenic signaling as EGF and TGFα (29–31). However, the kinetics of EGFR activation in response to these growth factors has not been studied in detail. To investigate this issue, HER-14 cells were serum-starved and treated with saturating concentrations of EGF, TGFα, or E4T for various periods of time followed by evaluation of the tyrosine phosphorylation status of the EGFR. As seen in Fig. 1A (and data not shown), the tyrosine phosphorylation of the 180-kDa species of the EGFR is comparable for all growth factors, consistent with the use of saturating ligand concentrations which lead to similar cell surface receptor occupancy levels. In addition, Fig. 1A and B show that in response to EGF stimulation the anti-phosphotyrosine antibody immunoreacted with high molecular weight species of the EGFR, represented by the smear above the 180-kDa band.

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ics of EGFR ubiquitination. EGF induced peak EGFR ubiquitination at 15-min post-receptor activation, whereas TGFα and E4T induced peak receptor ubiquitination at 5 min, when a large fraction of stimulated EGFRs is already internalized, as previously demonstrated (30). Furthermore, and in contrast to the prolonged presence of the tyrosine phosphorylated 180-kDa EGFR species (Fig. 1A), the ubiquitinated EGFR rapidly declined upon prolonged stimulation.

As it is formally possible that the ubiquitinated proteins observed in anti-EGFR (528) immunoprecipitates are derived from proteins that coprecipitate with the EGFR, we further denatured primary anti-EGFR immunoprecipitates by boiling in 1% SDS. Denatured proteins that were released into the supernatant were then used for recapture experiments with anti-EGFR (LA22 and clone 13) antibodies that recognize EGFR proteins under denaturing conditions (e.g. during immunoblotting). The anti-EGFR mAb 528 cannot be used for recapture experiments (data not shown) as it recognizes a conformational epitope in the extracellular domain of the EGFR that is destroyed upon denaturation. As illustrated in Fig. 1E, after stimulation of HER-14 cells with EGF, ubiquitinated species were still observed in anti-EGFR recapture experiments of denatured primary anti-EGFR immunoprecipitates, demonstrating that the >180 kDa ubiquitinated proteins indeed represent ubiquitinated EGFRs, consistent with earlier studies (11). It is important to note that the decreased ubiquitin signal observed in the secondary recapture (LA22 or c13) immunoprecipitates relative to the primary anti-EGFR (528) immunoprecipitates (Fig. 1E, upper panel) correlates with a decreased 180-kDa EGFR signal (Fig. 1E, lower panel), indicating that recapture of the EGFR is not quantitative. As excess amounts of antibody were used during the recapture (data not shown), we suggest that inefficient recapture of the denatured EGFR is due to poor recognition of the denatured EGFR by these antibodies. It is also noteworthy that, although Eps15 and Cbl can associate with the EGFR, the ubiquitinated species of these proteins run well below the 180-kDa mark, indicating that it is highly unlikely that ubiquitinated forms of these proteins contribute significantly to the observed ubiquitin signal in EGFR precipitates.

The data presented in Fig. 1 suggest that although EGF, TGFα, and E4T do not differ in the phosphorylation of the 180-kDa species of the EGFR, they differ markedly in inducing phosphorylation of the high molecular weight smear of the EGFR. The data also demonstrate that the superagonist E4T and TGFα induce markedly decreased EGFR polyubiquitination compared with EGF. These findings are consistent with the model that TGFα and E4T rapidly dissociate from internalized EGFRs due to acidification of endosomal vesicles, leading to a very transient EGFR ubiquitination, whereas EGF, which remains bound to the EGFR along the endocytic route, leads to more prolonged and enhanced EGFR ubiquitination.

**E4T and TGFα Induce Diminished Cbl Recruitment to Activated EGFR**—Efficient ubiquitination of the EGFR requires the recruitment of Cbl adapter proteins to phosphorylated tyrosine 1045 in the C-terminal tail of the EGFRs (13) and Cbl interaction with E2 ubiquitin-conjugating enzymes (22, 23). To demonstrate EGFR/Cbl interaction in HER-14 cell system, cells were treated with EGF for 5 min or left untreated. Fig. 2A shows that the addition of EGF resulted in co-precipitation of Cbl with the EGFR. To demonstrate Cbl-b interaction with activated EGFR, HER-14 cells were stimulated with EGF or left unstimulated. Fig. 2A shows that the EGFR coprecipitates with Cbl-b and with Cbl only in EGF-stimulated cells. Since TGFα and E4T induced decreased EGFR ubiquitination compared with EGF, we argued that this might be a direct result of poor recruitment of Cbl adapter proteins to TGFα- and E4T-stimulated EGFRs. In order to test this hypothesis, HER-14 cells were stimulated with either EGF, TGFα or E4T for the indicated period of time, followed by immunoprecipitation of Cbl proteins and subsequently immunoblotted with anti-phosphotyrosine antibody. The same blot was then re-probed with anti-c-Cbl antibody (lower panel). C, graph resulting from densitometric scanning of the phospho-EGFR signal (in MPV) relative to the c-Cbl signal (in MPV) as shown in Fig. 1B in response to EGF (solid line, diamonds), TGFα (dashed line, squares), and E4T (dotted line, triangles).
observed between EGF, TGFα, or E4T. The data presented in Fig. 2 indicate that Cbl adapter proteins interact with the activated EGFR in response to EGF, TGFα, and E4T and that these ligands induce similar levels of tyrosine phosphorylation of Cbl adapter proteins. In addition, the data also show that Cbl recruitment to the activated EGFR is less efficient in TGFα and, in particular, E4T-stimulated cells compared with EGF, which correlates with decreased EGFR ubiquitination levels induced by these growth factors.

E4T and TGFα Induce Decreased EGFR Degradation—It has been shown that TGFα and E4T binding to the EGFR is more susceptible to the low pH environment of the endosomal system when compared with EGF (30). Consistently, at saturating concentrations, E4T and TGFα promote enhanced receptor recycling compared with EGF, which can be blocked by the carboxylic ionophore monensin (30). Because E4T and TGFα stimulation resulted in decreased receptor ubiquitination and Cbl recruitment when compared with EGF (see Figs. 1 and 2), the possibility arises that TGFα and E4T might provoke lower rates of receptor degradation. To determine the kinetics of EGFR degradation induced by EGF, TGFα, and E4T, the expression level of EGFR in HER-14 cells was analyzed after stimulation with these growth factors for the indicated period of time (Fig. 3). EGFR stimulation for 5 h resulted in significant loss of the EGFR compared with unstimulated cells. The decline in the intensity of the 180-kDa band was paralleled by the appearance of antibody-reactive low molecular weight fragments at 3- and 5-h post-EGF treatment, which likely represent intermediate proteolytic EGFR products. In contrast, TGFα and E4T provoked low rates of receptor turnover at the same time points as EGF (shown in Fig. 3A and quantified in Fig. 3B), and intermediate proteolytic products were hardly seen (Fig. 3A). These findings demonstrate that decreased EGFR ubiquitination (Fig. 1), and decreased Cbl recruitment (Fig. 2) in response to TGFα and E4T is associated with decreased EGFR degradation, when compared with EGF. These results are also in accordance with our previous studies that TGFα and E4T induce enhanced EGFR recycling compared with EGF, which efficiently targets the EGFR for degradation (30).

Proteasome Inhibitor Lactacystin Fails to Block EGFR Degradation—Several studies have demonstrated that degradation of the EGFR is mediated by lysosomal proteases (5, 10, 24). However, accumulating evidence indicates that the degradation of the EGFR can be blocked by the proteasome inhibitor MG132 (16, 25), (data not shown). However, MG132 is not a specific inhibitor of proteasomal proteases. Thus, the involvement of proteasomes in EGFR degradation is still an issue of debate. To investigate this issue, bafilomycin A1 and lactacystin were used as lysosomal and proteasomal inhibitors, respectively. Bafilomycin A1 is a specific inhibitor of the proton pump V-type ATPase, which prevents the acidification of the endosomal compartments required for the maturation of lysosomal proteases. In addition, bafilomycin treatment entraps internalized surface molecules in late endosome MVBs, which precludes their transport to the lysosome, but it does not interfere with the localization of these molecules to early endosomes or late MVBs (33, 34). In contrast to MG132, lactacystin is a specific inhibitor of mammalian proteasomes (35, 36). As a result of lactacystin treatment, chymotrypsin-like, trypsin-like, and caspase-like peptidase activities are inhibited.

To study the mechanism of EGFR degradation, HER-14 cells were pre-treated with lactacystin or bafilomycin prior to stimulation with either EGF, TGFα or E4T for 6 h. The level of EGFR expression was analyzed by Western blotting. As seen in Fig. 4A, EGF stimulation in the absence of any inhibitor resulted in the degradation of the EGFR as expected. When lactacystin was added to the cells, a relatively small stabilizing effect on the EGFR was observed relative to unstimulated control cells (compare 2nd and 6th lanes). Increasing the concentration of lactacystin in the medium up to four times had no additional effects on the protection of full length EGFR (data not shown). Lactacystin treatment was effective, however, as it resulted in markedly increased accumulation of ubiquitinated cellular proteins (Fig. 4B), indicating that lactacystin treatment did interfere with proteasomal activity in HER-14 cells.
In contrast to lactacystin, bafilomycin treatment prevented the degradation of the EGFR completely (compare lanes 2 and 4). Consistent with Fig. 3, EGFR degradation in response to either TGFα or E4T in the absence of any inhibitor was markedly reduced compared with EGF (Fig. 4A). Lactacystin treatment of TGFα- and E4T-stimulated cells resulted in a relatively limited accumulation of full-length EGFR, when compared with EGF. In contrast to lactacystin, bafilomycin efficiently protected full-length EGFR in TGFα and E4T-stimulated cells (compare lanes 2 and 4). As the kinetics of EGFR degradation is somewhat delayed in the presence of lactacystin, these data indicate that proteasome activity may facilitate EGFR degradation, but clearly demonstrate that proteasome activity is not required for EGFR degradation.

**Lactacystin Blocks Ligand-induced c-Cbl Degradation**—It has been reported that Cbl adapter proteins undergo ligand-dependent ubiquitination and subsequent degradation, which can be blocked by lactacystin or MG132 (16). However, on the basis of electron microscopic co-localization studies, it has also been suggested that Cbl proteins remain associated with the EGFR in internal vesicles of MVBs (37). To determine the kinetics and underlying mechanism of c-Cbl degradation in response to EGFR treatment, HER-14 cells were treated with EGF for various time intervals and the level of c-Cbl expression was analyzed by means of immunoblotting with a polyclonal anti-c-Cbl antibody. As seen in Fig. 5A (middle panel), the addition of EGF resulted in a gradual decrease in the tyrosine phosphorylation of c-Cbl protein, which was paralleled by decrease of the immunoprecipitated c-Cbl (upper panel) (see also Fig. 2). The decrease in the c-Cbl expression level was also observed in total cell lysates (Fig. 5A, lower panel). Cbl expression levels remained low for 1–2 h of EGF stimulation, after which c-Cbl expression levels gradually recovered after 5 h when Cbl expression levels reach base line levels and nearly all EGFRs are degraded (Fig. 5B). To determine the mechanism responsible for c-Cbl degradation, HER-14 cells were pretreated with lactacystin or bafilomycin, followed by stimulation with EGF for various times. Fig. 5C shows that bafilomycin treatment has no effect on the degradation of c-Cbl. In contrast, when lactacystin was added to the cells, full-length c-Cbl was completely protected from degradation. In addition, lactacystin treatment markedly stabilized the 120-kDa phosphorylated species of c-Cbl protein up to 4 h of EGF stimulation (Fig. 5D), whereas in bafilomycin-treated cells Cbl phosphorylation was comparable to mock-treated (Me2SO-treated) cells. Surprisingly, lactacystin treatment also resulted in transient stabilization of the c-Cbl-EGFR complex compared with lactacystin-free cells (Fig. 5D). As lactacystin treatment does not block EGFR degradation (Fig. 4), the enhanced c-Cbl-EGFR co-precipitation seen is most likely due to lactacystin induced c-Cbl stabilization. Indeed, the tyrosine-phosphorylated 180-kDa EGFR band eventually disappeared from c-Cbl immunoprecipitates, consistent with the inability of lactacystin to prevent EGFR degradation. Even though bafilomycin treatment protects the EGFR from degradation, it does not lead to enhanced co-precipitation of the EGFR in Cbl immunoprecipitates, most likely because it does not protect c-Cbl from degradation. Indeed, when cells are treated with lactacystin and bafilomycin, co-precipitation of the phosphorylated EGFR with Cbl is enhanced related to either treatment alone, especially at 4 h, when significant EGFR degradation occurs. Even in the presence of bafilomycin alone or bafilomycin with lactacystin, phosphorylation of c-Cbl and EGFR is decreased at 4 h, relative to 1 h, which may be due to dephosphorylation. These data demonstrate that c-Cbl adapter proteins undergo ligand-induced degradation that is dependent on proteasomal but not v-ATPase activity.
Bafilomycin A1 Fails to Protect the Ubiquitinated EGFR from De-ubiquitination—Having determined the effect of lactacystin and bafilomycin on EGFR and Csh expression levels, we next evaluated the effect of these inhibitors on ligand-induced EGFR post-translational modifications including tyrosine phosphorylation and ubiquitination. For this purpose, HER-14 cells were treated with lactacystin or left untreated prior to stimulation with EGF. The EGFR was then immunoprecipitated and, subsequently, immunoblotting was performed with anti-phosphotyrosine antibody. Fig. 6A (top panel) shows that lactacystin induces limited accumulation of the 180-kDa tyrosine-phosphorylated EGFR when compared with lactacystin-free cells, reminiscent of the effect of lactacystin treatment on EGFR expression levels (Fig. 6A, lower panel). Indeed, lactacystin treatment did not prevent the appearance of EGFR degradation products (Fig. 6A, middle panel). Next, the effect of bafilomycin treatment on the level of the tyrosine phosphorylated EGFR was studied by incubating HER-14 cells with bafilomycin prior to stimulation with EGF. As seen in Fig. 6B (top panel), in the absence of bafilomycin the level of tyrosine-phosphorylated EGFR returned to the initial level after 4 h of stimulation with EGF, which is accompanied by the appearance of intermediate EGFR degradative products (Fig. 6B, middle panel) and decreased EGFR expression levels in total cell lysates (Fig. 6B, lower panel). In contrast, the addition of bafilomycin resulted in marked stabilization of the tyrosine phosphorylation of the 180-kDa species of the EGFR up to 4 h of EGF stimulation when compared with bafilomycin-free cells (Fig. 6B, top panel). We also noticed a more intense anti-phosphotyrosine staining of the high molecular weight smear in the EGFR immunoprecipitates. Nevertheless, even in bafilomycin-treated cells the EGFR phosphotyrosine content decreased slowly, suggesting that the EGFR is, at least in part, dephosphorylated by tyrosine phosphatases prior to its lysosomal degradation. Interestingly, during the early stages of EGFR activation, the apparent expression level of the 180-kDa EGFR band in whole cell lysates decreased similarly in both bafilomycin-treated and bafilomycin-free cells (Fig. 6B, lower panel). However, the apparent expression level of the EGFR recovered gradually in bafilomycin-treated cells but not in control cells. In theory, the decrease in the 180-kDa band of the EGFR during initial stages of receptor activation could be due to either degradation or ubiquitination. Because bafilomycin completely blocks the degradation of the EGFR we conclude that the transient decrease in the 180-kDa band is not due to receptor degradation, but to polyubiquitination, which shifts the EGFR to high molecular weight positions on the blot.

To directly assess the effect of lactacystin and bafilomycin on EGFR ubiquitination, HER-14 cells were preincubated with either MeSO, lactacystin, or bafilomycin prior to stimulation with EGF. The EGFR was then immunoprecipitated and the ubiquitinated species were visualized by immunoblotting with polyclonal anti-ubiquitin antibody. Fig. 6C shows that in the absence of lactacystin, the ubiquitinated species of the EGFR have disappeared after 4 h of stimulation. This decrease in the ubiquitination coincides with EGFR degradation (lower panel). When HER-14 cells were pre-incubated with lactacystin, a transient stabilization of the ubiquitinated EGFR was observed (top panel). The decline in the ubiquitination of the EGFR in the presence of lactacystin is ultimately associated with EGFR degradation (lower panel). Next, the effect of bafilomycin treatment was analyzed by performing the same exper-
HER-14 cells were pretreated with either Me₂SO, lactacystin, or proteasomal degradation pathway. To investigate the dependence of ubiquitination in bafilomycin-treated cells. Thus, we conclude that proteasome activity is required for de-ubiquitination of the EGFR prior to its degradation in the lysosomes. However, as the specific proteasome inhibitor lactacystin does not block EGFR degradation, we further conclude that EGFR de-ubiquitination is not required for EGFR degradation.

**DISCUSSION**

The results presented here show that TGFα and the superagonist ligand E4T are poorly coupled to Cbl-mediated EGFR ubiquitination because (i) E4T and, to a lesser extent, TGFα, induce decreased co-precipitation of the tyrosine phosphorylated EGFR with Cbl when compared with EGF, (ii) E4T, and to a lesser extent, TGFα, induce weak EGFR ubiquitination when compared with EGF, and (iii) E4T and TGFα induce a slow rate of EGFR turnover. As E4T and TGFα are known to enhance EGFR recycling in NIH3T3 cells overexpressing the EGFR (30), these findings are consistent with the model that Cbl recruitment to and subsequent ubiquitination of activated EGFRs correlates inversely with the ability of EGF, TGFα, and E4T to induce receptor recycling. Nevertheless, in accordance with observation that EGF, TGFα, and E4T share similar \(K_d\) values (31), the tested growth factors induced comparable tyrosine phosphorylation of the 180-kDa EGFR species at saturating ligand concentrations.

We envision two mechanisms that might explain the poor recruitment of Cbl proteins to the activated EGFR. First, the enhanced pH sensitivity of TGFα and E4T binding to the EGFR might promote early dissociation of these growth factors from the EGFR within early endocytic compartments when endosomal pH values gradually decrease. As a result, proteins such as Cbl that are bound to the EGFR might disassemble and/or may not be recruited to the EGFR anymore. Indeed, Madshus and co-workers (25) recently reported that TGFα induces markedly reduced EGFR tyrosine phosphorylation at acidic pH, and poor recruitment of Cbl to endosomes when compared with EGF. Second, the enhanced association/dissociation rate constants of superagonist ligands such as E4T (31) might also contribute to disassembly of the ligand-receptor complex and its associated cytoplasmic signaling molecules. At present, we cannot determine the relative contribution of pH dependence of receptor binding versus enhanced association/dissociation rate constants for the observed decrease in Cbl recruitment, EGFR ubiquitination and EGFR degradation (this study) or for the increased EGFR recycling (29, 30) observed in response to E4T stimulation. Both mechanisms probably contribute, although enhanced association and dissociation of superagonist ligands from cell surface expressed EGFRs might be extremely important to limit ligand depletion at low ligand concentrations (29), and therefore explain the markedly enhanced mitogenic activity of the superagonist ligands under these conditions (29, 30).

The early dissociation of the ligand-receptor complex after EGFR internalization may limit post-translational modification and could lead to a reduced steady-state EGFR tyrosine phosphorylation and/or Cbl-mediated EGFR ubiquitination, particularly when such modifications have not yet reached their maximum levels shortly after internalization. Thus, our finding that EGF, TGFα, and E4T induce similar levels of tyrosine phosphorylation of the 180-kDa EGFR could be explained if maximal ligand-induced EGFR tyrosine phosphorylation occurs on the plasma membrane or shortly after internalization, when TGFα or E4T have not yet dissociated from the EGFR in acidifying endosomes. However, EGF-induced EGFR ubiquitination clearly continues to increase for at least 15 min, when a large fraction of EGFRs has already been internalized (30). Indeed, based on the finding that Cbl is

![Inhibition of EGFR de-ubiquitination by simultaneous bafilomycin and lactacystin treatment](Image 79x598 to 285x738)

**Fig. 7. Inhibition of EGFR de-ubiquitination by simultaneous bafilomycin and lactacystin treatment.** HER-14 cells were starved overnight followed by pre-treatment with either Me₂SO, 10 μM lactacystin, 0.25 μM bafilomycin A₁, or with 10 μM lactacystin plus 0.25 μM bafilomycin A₁ for 1 h. Following stimulation with 100 ng/ml EGF for either 1 or 4 h, cells were lysed on ice and whole cell lysates were subjected to immunoprecipitation against the EGFR followed by immunoblotting with anti-ubiquitin antibody (upper panel). The same blot was then stripped and reprobed with anti-EGFR (1065) antibody (middle panel). Post-nuclear fractions (WCIs) were immunoblotted with anti EGFR (1005) antibody (lower panel).

**Inhibition of EGFR De-ubiquitination by Simultaneous Bafilomycin and Lactacystin Treatment—Proteasome inhibitors lead to the accumulation of ubiquitinated cellular proteins (see also Fig. 4B). This well known finding suggests a functional coupling between proteasomal activity and de-ubiquitination of substrate proteins. Indeed, de-ubiquitination enzymes are known to associate with the regulatory subunit of the 26S proteasome (38). Under conditions of lactacystin treatment, ubiquitinated cargo proteins may remain bound to the 19S regulatory complex and clog up the de-ubiquitination and proteasomal degradation pathway. To investigate the dependence of EGFR de-ubiquitination on proteasomal activity, HER-14 cells were pretreated with either Me₂SO, lactacystin, bafilomycin, or lactacystin plus bafilomycin prior to stimulation with EGF. The EGFR was then immunoprecipitated and immunoblotting was performed with anti-ubiquitin antibody. As seen in Fig. 7, lactacystin had little effect on the ubiquitinated status of the EGFR at 1 and 4 h of stimulation when compared with Me₂SO-treated cells. Similar to previous results, bafilomycin treatment resulted in marked accumulation of ubiquitinated EGFRs when compared with Me₂SO and lactacystin-treated cells. Strikingly, pretreatment with bafilomycin plus lactacystin resulted in almost complete inhibition of the decline in the level of ubiquitinated EGFR demonstrating
Proteasome-dependent EGFR De-ubiquitination

recruited to EGFR containing endosomes, Levkowitz et al. (12) suggested that Cbl-mediated ubiquitination occurs predominantly in early endosomes, although additional studies have revealed that Cbl-mediated EGFR ubiquitination also occurs in cells that fail to internalize the EGFR due to overexpression of a dominant negative dynamin mutant (39). Thus, the decreased EGFR ubiquitination and Cbl recruitment observed in response to TGFα and E4T stimulation, likely results from early pH-dependent dissociation of TGFα and E4T in acidifying endosomes. This model is further supported by the finding that Cbl is not efficiently recruited to endosomes in response to TGFα (25). In addition, the dissociated EGFR receptor chains might be particularly vulnerable for attack by constitutively active tyrosine phosphatases and deubiquitinating enzymes leading to a further decrease in EGFR post-transcriptional modification. Indeed, even though bafilomycin fully protects the EGFR from degradation (Figs. 4, 6, and 7), we found that EGFR tyrosine phosphorylation and ubiquitination are, at least in part, transient in the presence of bafilomycin (Figs. 6 and 7). A similar observation was previously made for the FceRI receptor where ligand dissociation resulted in a rapid receptor de-phosphorylation and de-ubiquitination (40).

As ubiquitination appears to act as a sorting signal on endosomal cargo proteins for targeting to the MVB pathway, we hypothesize that the decreased EGFR ubiquitination we observed in HER-14 cells in response to saturating doses of TGFα or superagonist ligands, leads to the enhanced EGFR recycling that has been documented under these assay conditions (30). When EGFR cell surface levels are limiting in the presence of excess growth factor, decreased degradation and enhanced recycling of internalized EGFRs may lead to enhanced mitogenic signaling. It should be noted however, that HER-14 cells that overexpress the human EGFR show similar mitogenic responses to the saturating doses of growth factors that we have used throughout this study (30). Differences in the mitogenic potency of EGF, TGFα, and E4T on HER-14 or 32D1 cells are detected only at very low non-saturating doses of growth factor (0.1–1.0 ng/ml range) (29, 30), conditions that do not allow evaluation of the EGFR ubiquitination status.

The role of proteasomes in the degradation of internalized plasma membrane receptors is an issue of debate. Indeed, several studies have demonstrated that the peptide aldehyde proteasome inhibitor MG132 blocks EGFR degradation (16, 25) (data not shown). Cbl proteins have also been reported to be degraded in an MG132 sensitive pathway (16). However, MG132 is not a specific proteasomal inhibitor: MG132 efficiently inhibits cathepsin B (25), which is a lysosomal protease of the papain family (41) that selectively catalyzes the degradation of the EGFR in the lysosome (10). Here we demonstrate that lactacystin, a specific inhibitor of the proteasome, fails to block EGFR degradation, although we consistently observed that lactacystin treatment leads to a minor and transient protection of the tyrosine phosphorylated and/or ubiquitinated EGFR (Figs. 4, 6, and 7). Lactacystin treatment effectively blocked proteasome function, however, as (i) it caused a marked accumulation of ubiquitinated cellular proteins (Fig. 4), (ii) it caused marked stabilization of tyrosine phosphorylated c-Cbl proteins (which may still undergo dephosphorylation under these conditions) (Fig. 5), (iii) it rescued c-Cbl proteins from proteasomal degradation (Fig. 5), and (iv) it blocked EGFR de-ubiquitination in the presence of bafilomycin (Fig. 7; see below). Moreover, increasing the dose of lactacystin failed to protect the EGFR from degradation (data not shown). Consistent with these findings, Madhus and co-workers (25) failed to prevent 125I-EGF degradation with lactacystin treatment. Thus, our findings indicate that proteasome activity facilitates EGFR degradation, even though it is not absolutely required for EGFR degradation.

Although we cannot exclude the possibility that proteasome activity is required for degradation of a hypothetical protein to facilitate lysosomal degradation, our experiments suggest an alternative explanation for the role of proteasomes in the lysosomal degradation of internalized plasma membrane receptors. When lysosomal proteases are blocked by bafilomycin treatment, EGFR ubiquitination is transient even though EGFR degradation is completely blocked. Most importantly, when the proteasome was inhibited under these circumstances by simultaneous lactacystin treatment, the ubiquitinated species of the EGFR was stabilized for prolonged periods of time and no EGFR de-ubiquitination could be detected in our experiments (Fig. 7). Interestingly, Longva et al. (25) reported that lactacystin treatment decreased the translocation efficiency of the EGFR from the outer limiting membrane to internal vesicles of MVBs. Although the possibility exists that prolonged lactacystin pre-treatment leads to depletion of the cellular free ubiquitin pool and consequently reduced EGFR ubiquitination and lysosomal targeting, this is clearly not the case in our study (Figs. 6 and 7). This leads us to propose a working model in which the EGFR is subject to de-ubiquitination reaction that may facilitate the sorting of EGFRs into internal vesicles of MVBs. As depicted in Fig. 8, the model presented in this work illustrates the role of proteasomes in the EGFR degradation pathway. EGFR activation triggers the recruitment of Cbl adapter proteins, which promote EGFR ubiquitination. After internalization, EGFR containing endocytic vesicles fuse with early endosomes. Prior to incorporation of EGFRs into internal vesicles of MVBs late endosomes, proteasomes mediate the removal of ubiquitin chains from the EGFR. Fusion of MVBs with pre-existing lysosomes ultimately leads to EGFR degradation. This de-ubiquitination process might be required for maintaining the level of free ubiquitin and may also enhance the entrance of the EGFRs into the internal vesicles of the MVBs. The proteasomes also catalyze the degradation of c-Cbl adapter proteins, an event that could be blocked by lactacystin treatment. Testing this working hypothesis will require the identification of the deubiquitinating enzyme(s) that is (are) responsible for EGFR de-ubiquitination.

Intriguingly, this working model is surprisingly similar to the vacuolar targeting of ubiquitinated cargo proteins in yeast, whereby the proteasome-associated deubiquitinating enzyme Doo4 removes ubiquitin from cargo proteins prior to their entry into internal vesicles of MVBs (42–45). Thus, in yeast, the Fur4 plasma membrane receptor accumulates in a non-ubiquiti-
nated form in a pep4 (a vacuolar protease) deletion strain and in a ubiquitinated form in a pep4 doa4 double mutant (45). Because doa4 deletion strains display decreased levels of free ubiquitin (44), resulting in decreased ubiquitination and vacuolar targeting of cargo proteins (43), removal of ubiquitin from lysosomal cargo proteins is in part responsible for maintaining cellular free ubiquitin pools. Importantly, in doa4 mutant cells that overexpress ubiquitin to supplement the decreased cellular pool of free ubiquitin, the Fur4 plasma membrane receptor is degraded (45), indicating that Doa4-mediated de-ubiquitination is not required for degradation of vacuolar cargo proteins. This is consistent with the fact that doa4 deletion strains do not display the characteristic Vps class E phenotype (44). In this study, we have demonstrated that the ubiquitination status of the EGFR exactly parallels that of the yeast Fur4 plasma membrane receptor under conditions where lysosomal/vacuolar degradation (compare bafilomycin treatment with pep4 strains) and/or de-ubiquitination (compare lactacystin treatment with doa4 strains) is blocked. Obviously, intricate regulatory mechanisms must exist to ascertain correct vacuolar/lysosomal targeting of ubiquitinated cargo proteins while simultaneously ensuring that ubiquitin moieties are recycled from cargo proteins for future use prior to their complete destruction in vacuoles/lysosomes. It is presently unclear, however, how proteasomes contribute to the de-ubiquitination of the EGFR without its concomitant proteasomal destruction. In this context it is intriguing to note that EGFR polyubiquitination appears to involve attachment of multiple mono-ubiquitin chains rather than (multiple) polyubiquitin chains (46, 47), which are normally required for proteasomal destruction.

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