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Epidermal Growth Factor Receptors with Tyrosine Kinase Domain Mutations Exhibit Reduced Cbl Association, Poor Ubiquitylation, and Down-regulation but Are Efficiently Internalized

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Abstract

Some non–small cell lung cancers (NSCLC) with epidermal growth factor receptor (EGFR) tyrosine kinase domain mutations require altered signaling through the EGFR for cell survival and are exquisitely sensitive to tyrosine kinase inhibitors. EGFR down-regulation was impaired in two NSCLCs with EGFR tyrosine kinase domain mutations. The mutant receptors were poorly ubiquitylated and exhibited decreased association with the ubiquitin ligase Cbl. Over-expression of Cbl increased the degradation of EGFR. Treatment with geldanamycin, an inhibitor of the chaperone heat shock protein 90, also increased both wild-type and mutant EGFR degradation without affecting internalization. The down-regulation of the mutant EGFRs was still impaired when they were stably expressed in normal human bronchial epithelial cells. Thus, the mutations that altered signaling also decreased the interaction of EGFRs with the mechanisms responsible for endosomal sorting.

Introduction

The epidermal growth factor (EGF) receptor (EGFR) plays a central role in the progression of many cancers, making EGFR an important target for cancer therapy. A subset of patients with non–small cell lung cancer (NSCLC) exhibit a substantial clinical response to the EGFR tyrosine kinase inhibitor gefitinib (Iressa, AstraZeneca Pharmaceuticals; refs. 1,2).

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Interestingly, a group of EGFR mutations within the tyrosine kinase domain acquired by tumors (and often amplified) in NSCLC patients have been associated with dramatic clinical responses to gefitinib or erlotinib (Tarceva, OSI/Genentech; refs. 3–5). Most recent estimates indicate that in unselected NSCLC samples EGFR mutations are present in 10% of cases in North America and Europe, but in ~30% to 50% cases in patients of East Asian descent (6). Such mutations, which are also more frequent in lifetime never smokers and females, include small in-frame deletions and point mutations within the ATP-binding pocket and cause significant abnormalities in the signaling behavior of the receptor (7). This perturbed signaling seems to explain the mutant receptor susceptibility to inhibitors, such as gefitinib. For example, the two most common mutations, the deletion L747-P753 and point mutation L858R, preferentially activated cell survival pathways mediated by Akt and signal transducer and activator of transcription, but not proliferative pathways mediated by extracellular signal-regulated kinase (8). Furthermore, RNA interference–mediated depletion of these mutant EGFRs caused extensive apoptosis, suggesting that the cells have become dependent on the survival pathways induced by the mutant receptors (8). Other studies have also reported aberrant EGFR signaling in cells with similar mutations (3,9,10).

In addition to activating signaling pathways, ligand binding by receptor tyrosine kinases (RTK), such as EGFR, also leads to their down-regulation. After binding ligand, the EGFR dimerizes and becomes phosphorylated. One of the phosphorylation sites provides a docking site for the ubiquitin ligase Cbl, which, together with an ubiquitin-loaded E2 enzyme, adds ubiquitin to specific lysine residues (11–13). Whether ubiquitylation is absolutely required for receptor internalization is not clear, but it does seem to be sufficient for internalization in the absence of any other sequence information in the receptor cytoplasmic tail (11,14,15). Activated EGFRs are rapidly internalized by clathrin- and/or caveolin-mediated endocytic processes (16). After internalization, endocytic vesicles fuse with early/sorting endosomes where, in contrast to receptors, such as the transferrin receptor that recycle, ubiquitylated EGFRs are sorted into endosomal intraluminal vesicles and eventually degraded. This occurs by a process believed to involve the recognition of ubiquitin by hepatocyte receptor substrate (Hrs) and the signal-transducing adaptor molecule (STAM; refs. 17,18). Hrs and STAM control the recruitment of other protein sorting complexes, such as ESCRT-I, ESCRT-II, and ESCRT-III that eventually deliver EGFRs into the luminal vesicles of multivesicular endosomes for transport to the lysosome (19).

Lung cancer cells that are dependent on chronic aberrant EGFR signaling, such as those expressing EGFRs bearing the L747-P753 deletion or the L858R mutation, must have mechanisms that allow the mutant receptors to avoid the acute down-regulation associated by receptor activation. In principle, this could be caused by changes in receptor recognition by the cellular machinery responsible for internalization and/or the mechanisms responsible for sorting into multivesicular endosomes. Recently, Yang et al. (20) reported that EGFRs bearing point mutations L858R or L861Q are refractory to down-regulation when expressed in 32D mouse hematopoietic cells and that this was associated with impaired ubiquitylation and increased binding by heat shock protein 90 (HSP90). Interestingly, the HSP90 inhibitor geldanamycin accelerated down-regulation of the mutant EGFRs. In this work, we report that two common types of tumor-acquired EGFR tyrosine kinase domain mutations, EGFR deletion E746-A750 and EGFR L858R, show impaired down-regulation in NSCLC cells bearing these proteins as well as when expressed in normal human bronchial epithelial cells (HBEC). These mutant EGFRs are not properly ubiquitylated by Cbl after binding EGF. In contrast to Yang et al. (20), we do not find a stable association of Cbl with the mutant receptors but increased association after EGF stimulation. We find similar effects of geldanamycin for increasing degradation of wild-type (WT) and mutant EGFRs and no impairment of mutant receptor internalization, suggesting that the defect in ubiquitylation is important for failure of the mutant receptors to sort into multivesicular endosomes.

Materials and Methods

Reagents and Antibodies

EGF, transferrin, and secondary antibodies conjugated to Alexa dyes were from Invitrogen and were used at concentrations recommended by the supplier. Protein A-Sepharose beads were from Amersham Biosciences. Monoclonal anti-EGFR (BD Biosciences), monoclonal anti-tubulin (Sigma-Aldrich), monoclonal and polyclonal anti-HA (Covance), monoclonal antiubiquitin (Santa Cruz Biotechnology), monoclonal anti-Cbl (BD Biosciences), and cetuximab were obtained from commercial sources. Monoclonal anti-EGFR conjugated to Alexa Fluor 546 was a gift from Steven Wiley (Pacific Northwest National Laboratory, Richland, WA). Plasmid encoding HA-tagged Cbl was provided by Joachim Herz (The University of Texas Southwestern Medical Center at Dallas, Dallas, TX). Recombinant EGF was purchased from Sigma-Aldrich. Protease inhibitor cocktail Complete was from Roche. Geldanamycin was from AG Scientific, Inc.

Cell Culture and Plasmid Transfections

NSCLC and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum. HeLa cell medium was also supplemented with 1 mmol/L sodium pyruvate and 10 mmol/L HEPES. HBECs were grown in keratinocyte serum-free medium (SFM; Invitrogen) that includes EGF and pituitary extract as supplements. Plasmid transfections were done using LipofectAMINE 2000 essentially following the manufacturer's instructions (Invitrogen). The only modification was that the amount of transfection reagent was 1.0 μ L, instead of the 1.5 suggested, per well of a 24-well plate. HBECs stably transfected with EGFR WT or mutant EGFR vectors have been described (21).

Receptor Down-Regulation and Internalization

Down-regulation—In preliminary experiments, we tested a range of concentrations of EGF to determine the concentration that would induce the maximum rate of EGFR down-regulation. Thirty to 100 ng/mL EGF gave essentially the same rate of EGFR degradation in HeLa cells, a concentration range used by many laboratories to study aspects of EGFR function (3,8,9, 11,12). For experiments measuring down-regulation, 90% confluent HeLa or NSCLC cells in six-well plates were serum starved overnight followed by addition of prewarmed EGF (100 ng/mL) in DMEM. HBECs were incubated overnight in keratinocyte SFM lacking EGF followed by stimulation with EGF (100 ng/mL) in the same medium. Cells were then incubated at 37°C for up to 4 h. At the times indicated in the figures, cell samples were lysed in sample buffer and cell extracts were resolved by SDS-PAGE and immunoblotted for EGFR and tubulin (loading control). Immunoblots were quantified by densitometry (Molecular Dynamics).

Internalization—Cells were cultured and starved as above, treated with EGF (100 ng/mL) for the times indicated in the figure legends, and then chilled on ice. Cell samples were washed with ice-cold PBS before labeling with a primary antibody against the extracellular domain of the EGFR that does not compete with ligand binding and a secondary antibody conjugated to Alexa diluted in PBS + bovine serum albumin (BSA; 0.2%) for 1 h on ice, respectively. After washing with ice-cold PBS, cells were detached with a nonenzymatic reagent (Sigma-Aldrich) to prevent removal of surface receptors, and fluorescence intensity was measured by flow cytometry using a FACSCalibur from BD Biosciences.

Receptor Ubiquitylation and Immunoprecipitation

Cells in 10-cm plates were cultured as described but treatment with EGF was for 10 min. Cells were then chilled on ice, washed twice with ice-cold PBS, and lysed in 50 mmol/L Tris (pH 8.0), 1% NP40, and 0.1% SDS containing a cocktail of protease inhibitors (Roche). Cell lysates

were centrifuged for 30 min at 4°C at $20,800 \times g$ to clear cellular debris before being immunoprecipitated with anti-EGFR antibody (from either BD Biosciences or Sigma-Aldrich) or cetuximab and protein A-Sepharose beads for 4 h. Immunoprecipitates were washed twice with 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.25% gelatin, 5 mmol/L EDTA, 0.02% NaN_3 , and 0.05% NP40 and once with 10 mmol/L Tris (pH 8.0) followed by SDS-PAGE and immunoblotting for the appropriate antibodies.

Membrane Fractionation

EGF-treated or untreated (control) HBECs in 10-cm plates were chilled on ice, washed twice with ice-cold PBS, and collected with 1.0 mL of 0.2 mol/L sucrose buffered with HEPES (20 mmol/L; pH 7.3) using a scraper. Cells were homogenized by 25 strokes in a prechilled steel homogenizer and centrifuged at $960 \times g$ for 15 min at 4°C. The supernatant was then recentrifuged at $128,000 \times g$ for 60 min at 4°C. Supernatants were then separated and the pellets were resuspended in sucrose buffer by brief sonication.

Fluorescence Microscopy

Cells on coverslips were washed with PBS and fixed in 3.7% formaldehyde for 15 min. The fixative was removed and cells were washed with DMEM and permeabilized with 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.25% gelatin, 5 mmol/L EDTA, 0.02% NaN_3 , 0.05% NP40, and 0.05% Triton X-100 or with methanol for 10 min at -20°C . Nonspecific binding sites were blocked with PBS containing 1% BSA before incubating samples with primary and secondary antibodies for 1 h at room temperature. Coverslips were mounted on glass slides with Aqua-Polymount (Polysciences) and viewed with a Zeiss Axiovert 200M microscope. For quantification of fluorescent EGF, parameters were set on the Axiovert 200M microscope and ORCA-285 (Hamamatsu) camera such that no area of any image was saturated (out of the linear range) and a series of images was taken with these settings. The outlines of cells to be quantified were delineated and the average pixel intensity within each cell obtained using OpenLab software version 4.0.2 (Improvision, Inc.).

Results

NSCLC cells with tyrosine kinase domain mutations exhibit impaired ligand-induced EGFR degradation

To examine the down-regulation of EGFRs after ligand binding, we treated HCC827 (deletion E746-A750), NCI-H3255 (missense L858R), NSCLC H157, (WTEGFR), and HeLa cells with EGF at 37°C for 0 to 4 h and then analyzed EGFR protein levels by immunoblotting. HeLa cells down-regulated EGFRs efficiently; the majority (~ 80%) of EGFRs were degraded after 1 h of EGF treatment (Fig. 1). H157 down-regulated EGFR somewhat slower than did HeLa, with ~ 60% of EGFR degraded after 2 h (Fig. 1A). By contrast, both NSCLC cell lines containing mutant EGFR exhibit much slower down-regulation of EGFRs after ligand binding. After 4 h of EGF treatment, NSCLC cells retained ~ 50% of EGFRs (Fig. 1A).

The slower degradation of EGFR in the NSCLC cells could be due to impaired receptor internalization or impaired sorting away from recycling membranes. To investigate these possibilities, we labeled EGFRs with a monoclonal antibody (mAb) that recognizes the extracellular domain and then followed their localization after EGF treatment. We also labeled cells with fluorescent transferrin, a marker for the endocytic recycling pathway. In HeLa cells, we observed that EGFRs are internalized following stimulation and after 25 min were located in vesicles that are distinct from those containing recycling transferrin (Fig. 1B), which is a sign of efficient sorting in endosomes (22). EGFRs in HCC827 cells also appeared in vesicles after 25 min and a significant fraction of these contained transferrin, indicating that EGFRs in these cells remained in vesicles that can recycle (Fig. 1B). Control experiments showed that

very little antibody is internalized before addition of EGF and that sorting of internalized EGFR from transferrin in the NSCLC H157 that expresses WT EGFR is efficient (Supplementary Fig. S1). We measured EGFRs at the cell surface by labeling cells with the same mAb before and after a 20-min treatment with EGF. For these experiments, in addition to the lung cancer cells with EGFR mutations HCC827 and H3255, we included cells of two additional lung cancer lines, H2126 and H157, which contain WT EGFR and do not exhibit defective EGFR down-regulation,⁷ as well as HeLa cells. EGFRs were removed from the surface of lung cancer cells HCC827 and H3255 efficiently and to a similar extent as from cancer cells expressing WTEGFR and just slightly less than HeLa cells (Fig. 1C). The combined results suggest that the slowed down-regulation of EGFRs in HCC827 and H3255 cells is most likely due to changes in the endosomal sorting of EGFRs, rather than internalization from the plasma membrane.

Ubiquitylation of EGFR is impaired in NSCLC cells with tyrosine kinase domain mutations

A molecular event that determines whether a signaling receptor will be recognized by the sorting machinery in endosomes for eventual degradation in the lysosome is ubiquitylation (23). Thus, we measured the ability of NSCLC cells to ubiquitylate EGFRs after EGF stimulation by immunoprecipitating EGFRs and immunoblotting with an ubiquitin antibody. In control HeLa cells, we detected significant ubiquitylation of EGFR after incubating 5 to 15 min with EGF at 37°C (Fig. 2). In contrast, ubiquitylation of EGFRs in HCC827 and H3255 cells was significantly reduced.

Mutations that alter EGFR signaling are also sufficient to inhibit down-regulation and ubiquitylation of EGFRs

Cells derived from lung cancer tumors, like other transformed cells, most likely present alterations in multiple pathways and such alterations could vary among different tumors. In addition, many NSCLC cell lines, including the ones used in this study, have amplified EGFR genes and overexpress the receptor (3). Thus, a potential explanation for the impaired EGFR ubiquitylation and down-regulation is that the cellular machinery has been saturated and cannot process the excess receptors. To investigate this possibility, we used immortalized (without viral oncogenes) HBECs derived from histologically normal lung tissue that stably express similar amounts of exogenous WT or the mutant EGFRs (21). In this way, we can examine the effect of the EGFR mutations in the same cellular background. We observed that HBECs expressing EGFR deletion E746-A750, or EGFR L858R, also had substantially slower down-regulation of EGFRs (Fig. 3A and B). Half-lives of 0.9, 5.8, and 4.7 for the WT, EGFR deletion E746-A750, and EGFR L858R, respectively, were obtained by fitting the data to one-exponential decay (Fig. 3B). Similarly, ubiquitylation of both mutant EGFRs in HBECs was also reduced compared with WTEGFR (Fig. 3C and D). The same result of impaired ubiquitylation was obtained using two different monoclonal anti-EGFR antibodies that recognize either the cytoplasmic (shown in Fig. 3) or the external domain (data not shown). Thus, these mutations seem to be sufficient to inhibit sorting of EGFRs into the lysosomal degradation pathway, presumably due to defective ubiquitylation.

EGFR tyrosine kinase domain mutations impair the EGF-dependent association of Cbl to EGFRs

The ubiquitylation of EGFR after ligand stimulation is carried out by the ubiquitin ligase Cbl. The EGFR phosphorylation site Tyr¹⁰⁴⁵ in the cytoplasmic domain provides the Cbl binding site and then Cbl monoubiquitylates the EGFR at several lysine residues (11,12,24). Thus, we tested whether Cbl was coimmunoprecipitated with EGFR after ligand stimulation in HBECs

⁷D. Padrón, unpublished data.

expressing WT or the mutant EGFRs. We detected an EGF-dependent coimmunoprecipitation of Cbl with WT EGFR in HBECs using cetuximab to precipitate EGFRs. However, in HBECs expressing EGFR deletion E746-A750 or EGFR L858R, we observed a 58.6% and 62.1% reduction in coimmunoprecipitation of Cbl with the receptors, respectively (Fig. 4). In addition, we also observed a minor amount of Cbl coprecipitating with mutated EGFR in unstimulated conditions, which was consistent with the minor degree of receptor ubiquitylation under those conditions shown in Fig. 3. We observed a similar defect in the association of Cbl with membranes as a response to EGF treatment. In serum-starved HBECs, Cbl is located primarily in the cytosol. After ligand stimulation, a larger fraction becomes membrane associated through the interaction with the EGFR. We detected an EGF-dependent increase in association of Cbl with a crude membrane fraction in HBECs expressing WT EGFR. The ratio of membrane (P)-associated Cbl to cytosol (S) increased 2.8-fold after adding EGF for 10 min. In contrast, the P/S ratio increased only 1.3- and 1.6-fold in cells expressing the mutant EGFRs (Supplementary Fig. S2).

Overexpression of Cbl accelerates degradation of EGFR in HCC827 cells

If the EGFR mutations impair the efficient association of Cbl with EGFR, perhaps by decreasing their binding affinities, it might be possible to overcome the defect in EGFR down-regulation by overexpressing Cbl. In fact, previous reports have shown that Cbl overexpression increases ligand-dependent degradation of RTKs, including EGFR and platelet-derived growth factor receptor (25,26). To attempt to correct the defect of slower degradation of EGFR deletion E746-A750 in HCC827 cells, we transiently transfected a plasmid encoding Cbl to enhance ubiquitylation of EGFRs. The low efficiency of transient transfection (~ 15%) of HCC827 cells precluded the use of a biochemical assay. Therefore, we used fluorescence microscopy to follow the effects of ligand binding by EGFRs in transfected cells overexpressing Cbl. In these experiments, the loss of EGFR was detected by the loss of internal fluorescent EGF as the ligand and receptor are both degraded. To determine the time course by which fluorescent EGF was cleared from cells, we incubated previously serum-starved HeLa cells with Alexa-labeled EGF for up to 3 h at 37°C. We observed that in HeLa cells, the signal decreased substantially after 1 h and was minimal after 2 h (25-min and 2-h time points are shown in Fig. 5A, *top*), suggesting that the receptor-ligand complex had reached the lysosome and had been degraded. By contrast, and consistent with the EGFR degradation data (Fig. 1), a substantial amount of fluorescent signal remained after 2 h in HCC827 cells (Fig. 5A, *bottom*). We then examined the extent of fluorescent EGF signal remaining after 25 min and 2 h in HCC827 cells overexpressing Cbl compared with that in neighboring untransfected cells. HCC827 cells overexpressing Cbl had consistently less signal than the untransfected cells after 2 h but not after 25 min (Fig. 5B, *top*). Using the imaging software OpenLab to measure pixel intensities, we found that the intensity of fluorescent EGF in Cbl-overexpressing cells ($n = 32$) was reduced ~ 45% ($P < 0.05$, t test) compared with neighboring untransfected cells ($n = 32$; see Fig. 5B, *bottom*).

Geldanamycin accelerates EGFR down-regulation but not internalization

A recent study reported that HSP90 bound constitutively to EGFR containing L858R or L861Q mutations and that this interaction was in fact responsible for slow EGFR down-regulation (20). We confirmed that in our assay conditions, EGFR with both the L858R and deletion E746-A750 mutations clearly associated with far more HSP90 than did WT receptors and that this association was not changed by treating cells with EGF (Supplementary Fig. S3). Yang et al. also reported that geldanamycin, an inhibitor of HSP90 function, accelerated ligand-induced degradation of the EGFR. We thus tested the effect of geldanamycin on EGFR down-regulation in our assay conditions using HBECs expressing either WT EGFR or receptors with the L858R or deletion E746-A750 mutations. First, we tested the efficacy of geldanamycin in inhibiting the interaction of EGFR with HSP90 by coimmunoprecipitation experiments. We found that

0.2 $\mu\text{mol/L}$ was sufficient to inhibit $\sim 50\%$ of the association of HSP90 with the EGFR.⁷ We observed that this concentration accelerated the down-regulation of mutant receptors ~ 2 -fold and increased down-regulation of WT receptors to some extent (Fig. 6). Finally, we measured internalization of WT and mutant EGFRs with and without geldanamycin treatment. We found that mutant receptors in HBEC cellular background were internalized at the same rate as WT receptors. Furthermore, geldanamycin treatment did not influence the kinetics of internalization of either WT or mutant EGFRs (Supplementary Fig. S4).

Discussion

Chronic activation of RTK-dependent pathways is generally recognized to be associated with cancer. More recently, however, it has been appreciated that impaired negative regulation of receptor signaling may also be an important mechanism involved in cancer (23,24,27). The ubiquitin ligase Cbl is considered a key regulator of down-regulation of RTKs, including the EGFR, by controlling receptor endocytosis and targeting to the lysosomal-degradative pathway. In fact, several oncogenic forms of RTK have been shown to escape Cbl-mediated degradation (27). In general, these proteins have mutations that remove the binding site for Cbl on the receptor leading to alteration of receptor down-regulation that is an independent mechanism for increasing RTK signaling. However, for any cancer that is dependent on sustained RTK signaling for cell proliferation and survival, including situations in which receptors have activating mutations, some mechanisms for escaping down-regulation must operate, or the activated receptors would simply be degraded. Therefore, we investigated the down-regulation of mutant EGFRs in two gefitinib-sensitive NSCLC cell lines, in which the mutations were outside the known Cbl interaction site.

We found that two mutations in the EGFR frequently observed in human tumors, L858R and deletion E746-A750, are each sufficient to impair EGFR ubiquitylation and down-regulation. The mutations were also sufficient to significantly reduce the EGF-dependent association of Cbl with EGFR. A recent study had found that EGFR mutations L858R and L861Q exhibited similarly impaired receptor down-regulation when expressed in mouse hematopoietic cells (20). Yang et al. (20) observed that the EGFR mutations L858R and L861Q associated with Cbl even in unstimulated conditions and saw no increased association after adding ligand. Our results differ from this study because we observed that very little Cbl coimmunoprecipitated with EGFR L858R and EGFR deletion E746-A750 in the absence of EGF (Fig. 4). On binding EGF, the mutant EGFRs bound 5- to 10-fold more Cbl, although this was only half that bound by the WT EGFR. We found a similar result in a different assay measuring the EGF-dependent association of Cbl to a crude membrane fraction. These results are consistent with the degree of ubiquitylation we observed (Fig. 3) and suggest that binding of Cbl to the mutant receptors is defective. The binding of Cbl to EGFR requires phosphorylation of Y1045. However, phosphorylation at Y1045 in nontransformed mouse mammary epithelial cells stably expressing EGFRs with L858R or deletion 747–753 mutations was similar to WT in a previous study (8). We tested phosphorylation of Y1045 under our assay conditions and found no decrease in EGFR L858R and a very modest decrease in EGFR deletion E746-A750.⁷ Nevertheless, we show that cells expressing these mutant EGFRs recruit less Cbl in response to EGF, suggesting that binding to Cbl or to another protein that cooperates with Cbl is impaired. Presumably, these mutations alter the cytosolic domain decreasing the affinity for binding Cbl without removing the binding site because we also show that increasing Cbl expression can at least partially compensate for the effect of the mutation on down-regulation.

Contrary to our results and those of Yang et al. (20), a recent study had reported that EGFR L858R or EGFR deletion E746-A750 expressed exogenously in H1299, a NSCLC cell line whose endogenous EGFR is not mutated, was degraded at a rate similar to exogenous WT EGFR in those cells (9). However, the WT receptor in H1299 cells is degraded as slowly as

the mutant receptors are when expressed in HBECs.⁷ Therefore, it is possible that H1299 cells contain defects in cellular machinery for receptor down-regulation that have the same effect for prolonging receptor half-life as do some receptor mutations. Our studies used a normal epithelial cellular background and Yang et al. (20) used hematopoietic cells lacking endogenous EGFRs where the effects of receptor mutations can be isolated from other possible defects in a cancer cell line. In a recent review, Bache et al. (23) compiled evidence that links deregulation of RTK degradation machinery with cancer, including alterations in Eps15, endophilin-2, Huntingtin interacting protein, and others required for receptor down-regulation. It will be interesting to learn if changes in such factors are also involved in lung cancer.

HSP90, a chaperone that plays a role in maturation and function of RTKs, has been reported to interact with EGFR containing tyrosine kinase domain mutations but not with WT receptors (20,28). However, we observed that treatment with geldanamycin, an inhibitor of HSP90, increased degradation of both WT and mutant EGFRs. Thus, either the small amount of HSP90 binding we detect for WTEGFR has consequences for receptor down-regulation, or HSP90 has effects on other cellular components that influence EGFR degradation. HSP90 binds to the EGFR heterodimerization partner ErbB-2 and restrains its signaling by limiting heterodimer formation (29). Therefore, we cannot rule out an indirect effect of geldanamycin through ErbB-2 on down-regulation of EGFRs in NSCLC cells or HBECs.

We did not observe defective internalization of EGFR L858R or EGFR deletion E746-A750 compared with WTEGFR and treatment with geldanamycin had no noticeable effect at this step. Our results indicate that the EGFR L858R and EGFR deletion E46-A750 are poorly ubiquitinated and degraded but efficiently internalized. A similar result was observed for an ubiquitylation-deficient mutant Met receptor (Y1003F), which was mistargeted for degradation but internalized with normal kinetics (14). Thus, if HSP90 association to mutant EGFR explains impaired down-regulation, the effect occurs after receptors are removed from the plasma membrane, presumably in the processes that sort EGFR into the intraluminal vesicles of multivesicular endosomes. This interpretation is consistent with the prolonged colocalization of EGF and transferrin receptors we observed in HCC827 cells.

Defective down-regulation of RTK has been increasingly linked to cancer. For example, components of the endocytic machinery have been found as fusion oncoproteins (30). In addition, regulators of receptor degradation other than Cbl have been linked to cancer, such as Tsg101, a component of the ESCRT-I complex (31,32). Currently, there are no rationally designed therapies available that target the down-regulation machinery for EGFR or other RTKs. Previous evidence indicates that down-regulation of EGFRs can be increased by either elevating expression of ubiquitin ligases or reducing ubiquitin hydrolases in cells such as Chinese hamster ovary or HeLa (25,33,34). We show that overexpression of Cbl accelerated degradation of EGFR in a NSCLC cell line, HCC827, suggesting that impaired ubiquitylation is a molecular defect responsible, at least in part, for impaired EGFR down-regulation in these cells. Future experiments examining whether factors that increase EGFR down-regulation have positive effects on NSCLC will be of special interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

NSCLC cells sensitive to tyrosine kinase inhibitors exhibit impaired ligand-induced EGFR down-regulation. NSCLC cell lines HCC827 and H3255, H157, and HeLa cells were serum starved overnight and then stimulated with EGF (100 ng/mL) for 0, 1, 2, 3, and 4 h before lysis in sample buffer. Cell extracts were then resolved by SDS-PAGE and immunoblotted with EGFR and tubulin antibodies. *A, top*, representative of three experiments; *bottom*, quantification by densitometry of the immunoblot. Values in each cell line are expressed as the percentage of EGFR at time zero of the same cell sample. Both HCC827 and H3255 NSCLCs containing EGFR tyrosine kinase domain mutations show prolonged EGFR levels compared with NSCLC H157 and human cervical carcinoma HeLa cells expressing WT EGFR.

B, cells on coverslips were serum starved overnight. HeLa and HCC827 cells were labeled with anti-EGFR antibody for 30 min at 37°C before the addition of prewarmed DMEM containing EGF (100 ng/mL) and Alexa-transferrin (50 µg/mL) for an additional 25 min. Cells were then chilled on ice, washed, and fixed in formaldehyde. Note that HCC827 with mutant EGFR fail to sort EGFR from transferrin (prolonged positive merged signal; see *arrows*) compared with HeLa cells. Bar, 10 µm. *C*, NSCLC cells with (HCC827 and H3255) or without (H2126 and H157) EGFR tyrosine kinase mutations and HeLa cells were serum starved overnight and then stimulated with EGF (100 ng/mL) for 0 and 20 min before chilling cells on ice. Cells were subsequently labeled with EGFR and Alexa-conjugated secondary antibodies on ice, resuspended with a nonenzymatic reagent, and analyzed by flow cytometry. The average fluorescent intensity of 10,000 cells treated with EGF after 20 min is plotted as a percentage of the average fluorescence of untreated cells. *Columns*, mean of data from three to five experiments; *bars*, SE.

Figure 2.

Ubiquitylation of EGFRs is impaired in NSCLC cells with EGFR tyrosine kinase domain mutations. HeLa, HCC827, and H3255 cells were serum starved overnight and stimulated with 100 ng/mL EGF for 10 min and EGFRs were immunoprecipitated. After SDS-PAGE, immunoprecipitates were immunoblotted for ubiquitin. A blot showing the amount of EGFR in the input samples and EGFR and ubiquitin in the immunoprecipitates is shown. Representative results from one of three experiments. Note the reduced ubiquitylation of mutant EGFRs in the NSCLCs after EGF stimulation compared with that of the WT receptor in HeLa cells.

Figure 3.

EGFR tyrosine kinase domain mutations are sufficient to inhibit down-regulation and ubiquitylation of EGFRs. HBECs stably expressing either exogenous WT, the deletion mutant (Δ746-750), or L858R mutant EGFRs were cultured in medium lacking EGF overnight and then stimulated with 100 ng/mL EGF for 0, 1, 2, 3, and 4 h and analyzed by immunoblotting with antibodies to EGFR and tubulin (as a loading control). *A*, an image of a representative blot. *B*, blots from three experiments were quantified by laser scanning densitometry and average values of the percentage of EGFR remaining are graphed. *Points*, EGFRs; *bars*, SE. Note that HBECs expressing mutant EGFRs maintain higher levels of receptors after ligand binding compared with HBECs stably expressing comparable amounts of WT EGFR. *C*, HBECs starved as above were also stimulated for 10 min with 100 ng/mL EGF and EGFR was immunoprecipitated as in Fig.2. Representative blot of the amount of EGFR in the starting lysate and amount of ubiquitin and EGFR in the immunoprecipitate. *D*, quantification of three similar experiments by densitometry. Values are expressed as a percentage of the ubiquitylated receptor in cells expressing WT EGFR in the absence of EGF. *Columns*, ubiquitylation; *bars*, SD. Note that mutant EGFRs exhibit significantly less ubiquitylation than WT EGFRs.

Figure 4.

EGFR tyrosine kinase domain mutations impair the EGF-dependent association of Cbl to EGFRs. HBECs expressing WT or mutant EGFRs were starved of EGF overnight and then stimulated for 10 min with 100 ng/mL EGF and EGFR was immunoprecipitated with cetuximab as indicated in Materials and Methods. *A*, representative blot of the amount of Cbl and EGFR in the immunoprecipitates. *B*, the graph presents the quantification of three experiments by densitometry. Cbl values were normalized to the amount of EGFR in the immunoprecipitates and are expressed as a percentage of the value in cells expressing WT EGFR (100%) in the presence of EGF. *Columns*, Cbl/EGFR ratio; *bars*, SE.

Figure 5. Increased Cbl expression accelerates EGF degradation in HCC827 cells. HCC827 cells on coverslips were transfected with a plasmid encoding HA-tagged Cbl and incubated overnight in serum-free medium. Transfected HCC827 and untransfected HCC827 and HeLa cells were then exposed to 10 μ g/mL of fluorescent EGF for 25 min, washed, and either fixed immediately or incubated for 2 h at 37°C in DMEM before fixation. **A**, EGF fluorescence in untransfected HCC827 and HeLa cells after 25 min or 2 h of chase. Bar, 10 μ m. **B**, transfected HCC827 cells were labeled with an anti-HA mAb to identify cells expressing Cbl. The graph shows the average pixel intensity of EGF fluorescence in cells overexpressing Cbl as percentage of EGF fluorescence of untransfected cells in the same sample. Values are the average of two and three

experiments for the 25-min and 2-h time points, respectively. *Columns*, internal fluorescence; *bars*, range (25-min sample) and SD (2-h sample). A combined total of 50 [25 of each transfected and untransfected (*Untransf*)] and 64 (32 in each transfected and untransfected) cells were measured for the 25-min and 2-h time point, respectively.

Figure 6. Geldanamycin accelerates EGFR down-regulation. HBECs stably expressing either exogenous WT, deletion mutant Δ E746-A750, or L858R mutant EGFRs were cultured in medium lacking EGF overnight and then stimulated with 100 ng/mL EGF for 0, 1.5, 3, and 4.5 h in the absence or presence of geldanamycin (0.2 μ mol/L). Cell samples were prepared and immunoblotted with EGFR and tubulin antibodies. *A*, an image of a representative blot. *B*, blots from three or four experiments were quantified by laser scanning densitometry and average values of the percentage of EGFRs remaining are graphed. *Points*, EGFRs; *bars*, SE.