c-Cbl-mediated Ubiquitinylation Is Required for Epidermal Growth Factor Receptor Exit from the Early Endosomes*

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Epidermal growth factor receptor (EGFR) controls cell growth and has a key role in tumorigenic processes. The extent of EGFR signaling is tightly regulated by post-transcriptional modifications leading to down-regulation of the levels of the receptor. Previous studies from our laboratory demonstrated that the reactive oxidant hydrogen peroxide activates the EGFR, yet, without down-regulation of the receptor levels, which results in prolonged receptor signaling. In this study, we examined the role of the E3 ligase c-Cbl, as a possible link between oxidative stress, EGFR signaling, and tumorigenic responses. First, we ectopically expressed a mutant EGFR (Tyr-1045→Phe) in cells lacking endogenous receptor, to determine whether the lack of phosphorylation at this site is the cause for EGFR retention. These findings suggest that abrogation of tyrosine 1045 phosphorylation alone is not enough to retain the EGFR at the plasma membrane under oxidative stress. Second, through the use of the Src inhibitor PP1, our findings establish EGFR movement out of the early endosomes as the exact location where c-Cbl-mediated ubiquitinylation is essential for EGFR trafficking. Finally, our studies substantiate the findings that c-Cbl-mediated ubiquitinylation is needed for degradation, but not for internalization of the EGFR in both transfection-dependent Chinese hamster ovary cells and transfection-independent A549 lung epithelial cells. These findings only begin to explain the features seen under oxidative stress, but they yield a greater understanding of the role of c-Cbl in EGFR trafficking.

Binding of epidermal growth factor (EGF)† to its receptor (EGFR) results in the activation of numerous cellular signaling pathways essential for cellular proliferation, survival, and differentiation. The length and intensity of these signals are controlled by several negative regulatory mechanisms (1), leading to complete signal inactivation by endocytosis and degradation of the receptor. Such a progression is required for eliminating constitutive signaling and tumorigenesis. Upon EGFR binding, the EGFRs are rapidly internalized from the cell surface through numerous pathways, including clathrin-coated pits (2). Internalized receptors are associated with early endosomes and then mature into late endosomes. In these bodies the EGFRs go through sorting and are either cycled back to the plasma membrane or transferred to the lysosomes for degradation (2–4).

Although much remains unknown about these processes, c-Cbl-mediated ubiquitinylation has been shown to be essential for regulating these events and ensuring proper degradation of EGFR (5–8). Upon EGFR induction, c-Cbl binds directly to the EGFR via Tyr-1045 (8) and indirectly through the SH3 domain of Grb2 (9). c-Cbl binding and its consequential phosphorylation results in the activation of the E3 ligase activity of c-Cbl, recruitment of the ubiquitin-conjugating enzyme UbcH7 (10), and EGFR ubiquitinylation.

Multiple studies have established that the oncogenic potential of the EGFR receptor is linked to its inability to undergo normal clathrin-mediated endocytosis and degradation (11, 12). We and others have shown that exposure to oxidative stress in the form of H2O2 results in a tyrosine-phosphorylated EGFR (13, 14) that is unable to undergo normal down-regulation (15). This aberrant phosphorylation does not support enhanced receptor turnover rate (14, 15), coincides with enhanced cell proliferation (16), and has been shown to facilitate tumor promotion processes in the rat liver epithelial cell line T51B (17).

To elucidate molecular mechanisms linking oxidative stress, EGFR signaling, and tumorigenic responses, we studied the role of c-Cbl-mediated ubiquitinylation under oxidative stress. Upon mapping the EGFR phosphorylation sites, we found that phosphorylation at Tyr-1045, the docking site for c-Cbl, (8) is abrogated under oxidative stress. Consequently, the EGFR fails to recruit the ubiquitin ligase, c-Cbl, and thus is not ubiquitinated and is not degraded (15). We, therefore, suggested that this deficiency might have a key role in linking oxidative stress, the EGFR, and tumorigenesis by conferring prolonged receptor signaling at the plasma membrane.

To gain a better understanding of how a receptor lacking c-Cbl binding may lead to tumorigenesis, we ectopically expressed a mutant EGFR (Tyr-1045→Phe) in CHO cells to determine whether the lack of phosphorylation at this site is indeed the cause for prolonged EGFR retention at the membrane under oxidative stress. Our findings suggest that the inability of the EGFR to bind c-Cbl under oxidative stress is not solely due to its abrogated Tyr-1045 phosphorylation because the Y1045F mutant is still able to bind c-Cbl, probably indirectly via Grb2.

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The abbreviations used are: EGF, epidermal growth factor; EGFR, EGFR receptor; MAP, mitogen-activate protein; E3, ubiquitin-protein isopeptide ligase; CHO, Chinese hamster ovary; WT, wild type; GO, glucose oxidase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; biotin-X-NHS, analog of biotin N-hydroxysuccinimide ester containing a 6-aminoacaproic acid the spacer arm; EEA1, early endosome-antigen 1; PP1, 4-amino-1-[4-tert-butyl-3-(1-naphthyl)]pyrazolo[3,4-d]-pyrimidine.
Additionally, to establish the exact location where c-Cbl-mediated ubiquitinylation is essential for EGFR trafficking, A549 airway epithelial cells (natural EGFR expressers) were exposed to the Src inhibitor, PP1, which prevents EGFR ubiquitinylation. We identified EGFR movement out of the early endosomes as the precise site where EGFR ubiquitinylation is required. Finally, our studies substantiate the findings that c-Cbl-mediated ubiquitinylation is needed only for degradation, but not for internalization in both transfection-dependent CHO cells and transfection-independent A549 lung epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—A549 human lung epithelial cells from ATCC (Manassas, VA) were maintained in F-12K (Knighn’s modification) nutrient mixture (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. To prepare cell lysates, A549 cells were from ATCC and maintained in F-12 Ham’s medium (Invitrogen), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Prior to experiments, cells at 50–60% confluence were serum-starved overnight in F-12K medium containing 0.5% dialyzed fetal bovine serum. CHO-K1 cells were from ATCC and maintained in F-12 Ham’s medium (Invitrogen), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Prior to experiments, cells at 50–60% confluence were serum-starved in F-12 Ham’s medium containing 0.5% dialyzed fetal bovine serum. CHO cells were stably cotransfected with expression vectors for human c-Cbl and oncogenic (Y1045F) mutant EGFR (both generously provided by Y. Yarden, Weizmann Institute, Israel) or WT EGFR, kindly provided by J. Schlessinger (Yale University). For other experiments, CHO cells were transiently transfected with either WT or mutant c-Cbl 381A expression vector (a generous gift from W. Y. Langdon, University of Western Australia).

**Treatments**—H₂O₂ was generated by adding glucose oxidase (GO) (type II from Aspergillus niger, 15,500 units/g, Sigma) to serum-free Dulbecco’s modified Eagle’s medium (Invitrogen) containing 25 mM glucose and 0.5% BSA. During experiments, cells were incubated for 15 min with medium that was pre-conditioned with GO for 15 min. For incubation periods greater than 15 min, GO-containing medium was replaced every 15 min with pre-conditioned medium. For GO treatment, cells were incubated in the same medium supplemented with 100 ng/ml EGF (Upstate Biotechnology, Inc., Waltham, MA). c-Cbl ubiquitin ligase activity was inhibited using PP1. Serum-starved A549 cells were pretreated with 5 or 50 μM PP1 (Biomol, Inc., Plymouth Meeting, PA) for 30–45 min. Cells were then treated as noted in the presence of PP1. To regain c-Cbl ubiquitin ligase activity, cells were then washed three times with PBS, followed by the addition of medium containing only EGF. For inhibition of recycling, cells were preincubated for 40 min with 100 μM monensin (Sigma) and treated as described.

**Lysate Preparation, Immunoprecipitation, and Western Blotting**—Lysate preparation and protein immunoprecipitation were performed as described by Bao et al. (18). After treatments, cells were extracted in solubilization buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, protease inhibitor mixture (Sigma), and phosphatase inhibitor mixture (Sigma). Lysates were cleared by centrifugation, and proteins in the lysate supernatants were immunoprecipitated by overnight incubation with the indicated antibodies at 4 °C, followed by protein A (Repligen Corp., Needham, MA) precipitation for 1–2 h. Precipitating antibodies used are as follows: anti-EGFR receptor clone C225 (a generous gift from ImClone Systems Inc., New York, NY); anti-Grb2 clone C-23 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-c-Cbl clone C-15 (Santa Cruz Biotechnology). Immunoprecipitates were washed three times with HNTG buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glyceral, resolved by gel electrophoresis, and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in Tris-buffered saline, pH 7.5, containing 0.5% Tween 20, 1% BSA, and 5% milk and then blotted overnight with primary antibodies followed by secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) linked to horseradish peroxidase. Immune reactive protein bands were detected with an enhanced chemiluminescence reagent (Pierce). Blotting antibodies used were as follows: anti-EGFR receptor RK2 (kindly provided by Dr. J. Schlessinger); anti-phosphotyrosine PY-20, anti-Grb2 clone C-23, anti-Src, and anti-c-Cbl C-15 antibodies from Santa Cruz Biotechnology; anti-Grb2 antibody P2G7 from Covance (Princeton, NJ); anti-phospho-Akt serine 473 antibody, anti-phospho-ERK1/2 (p44/42 MAP kinase) antibody, anti-phospho-Src Tyr-416 (Cell Signaling, Inc.); and anti-Akt antibody and anti-ERK1/2 (p44/42 MAP kinase) antibody (Cell Signaling Inc.).

**RESULTS**

**H₂O₂ Exposure and Tyr-1045 Mutation Confer Aberrant EGFR Degradation and Prolonged EGFR Signaling**—We have recently demonstrated that activation of the EGFR by reactive oxidants such as H₂O₂ fails to enhance c-Cbl-mediated normal receptor down-modulation. We proposed that abrogated phosphorylation of Tyr-1045 of the EGFR is the basis for this aberrant down-regulation, and it is a result of the inability of c-Cbl to bind to the EGFR Tyr-1045 and to ubiquitinate the EGFR.

To address this possibility further, we utilized a mutant EGFR in which Tyr-1045 had been replaced by phenylalanine (Y1045F) (8). This mutant was co-transfected with c-Cbl into Chinese hamster ovary (CHO) cells and compared with the wild type (WT) EGFR. We investigated the fate of surface receptors by using the water-soluble, membrane-impervious binetin-X-NHS, which labels accessible lysines of surface receptors (20, 15). Biotinylation was followed by immunoprecipitation of the EGFR and Western blot analysis as described before (15). As shown in Fig. 1A, WT EGFR-transfected cells exposed to EGF demonstrated a rapid drop in EGFR levels, whereas the Y1045F-transfected cells showed EGFR stability with unchanged levels at all detected time points up to 6 h of EGF treatment. Therefore, the cells containing the Y1045F mutant demonstrated a resistance to degradation, whereas the levels of the WT receptor dropped quickly in the presence of EGF. These results parallel our previous data that demonstrated that EGFR is resistant to degradation under H₂O₂ exposure (15).

Given that the Y1045F mutant showed a similar degradation phenotype to that of the WT receptor under oxidative stress, we were interested in investigating whether this inhibition of deg-
Fig. 1. The Y1045F mutant fails to be degraded under EGF treatment and confers prolonged signaling of Akt and ERK. A, cell surface molecules of serum-starved CHO cells transfected with c-Cbl and either WT EGFR or Y1045F EGFR were biotinylated as described under “Experimental Procedures,” followed by incubation with 100 ng/ml EGF for the indicated time intervals. The EGFR was immunoprecipitated from cell lysates using anti-EGFR C225 antibody, separated on SDS-PAGE, and immunoblotted with horseradish peroxidase-conjugated streptavidin, and anti-c-Cbl C-15 antibody. Left panel, enhanced chemiluminescence detection of biotinylated EGFR, and EGFR-associated c-Cbl. Right panel, the optical intensities of the corresponding bands were quantified, and the plotted data were used to estimate the amount of WT-EGFR and Y1045F remaining after EGF treatment. B, serum-starved CHO cells transfected with c-Cbl and either the Y1045F or WT EGFR were incubated with 100 ng/ml EGF or 1 unit/ml glucose oxidase (to generate H2O2) as described under “Experimental Procedures” for the indicated time points. Cells were lysed, separated by SDS-PAGE, and immunoblotted with anti-phosphotyrosine, PY20 antibody, anti-phospho-Akt serine 473 antibody, anti-phospho-ERK1/2 (p44/42 MAP kinase) antibody, anti-EGFR RK2 antibody, anti-Akt antibody, or anti-ERK1/2 (p44/42 MAP kinase) antibody. Blots shown are representative of three or more experiments. C, serum-starved CHO cells transfected with c-Cbl, and either the Y1045F or WT EGFR were incubated with 100 ng/ml EGF or 1 unit/ml glucose oxidase (to generate H2O2) for 15 min. Cells were lysed, separated by SDS-PAGE, and immunoblotted with EGFR RK2 antibody, anti-c-Cbl C-15 antibody, and anti-ubiquitin P4G7 to establish transfection and ubiquitination controls.

radiation also paralleled the increase in length of EGFR signaling as seen under H2O2 exposure. CHO cells transfected with c-Cbl and the Y1045F mutant or WT EGFR were analyzed for Akt and ERK activation using anti-phosphorylation-specific antibodies in Western blot analysis. Transfection levels of the EGFR, Y1045F mutant, are detailed in Fig. 1C. After 2-h treatment with EGF or H2O2 (generated via glucose oxidase (GO)), EGFR, Akt, and ERK phosphorylation all remained significantly stronger in Y1045F-transfected cells than in cells expressing the WT EGFR and treated with EGF (Fig. 1B). These results are similar to those observed following exposure to H2O2 (Fig. 1B). Yet, the signaling through Akt and ERK was much stronger upon treatment of H2O2 at both time points. Because EGF was administered in levels that saturate EGFR binding capacity, it is possible that the additional H2O2 signaling activates Akt and ERK through a more complex mechanism than through the EGFR alone.

Tyr-1045 Mutation Confers Slower Internalization of the EGFR—Because it was previously shown that EGFR activation under oxidative stress is not followed by c-Cbl-mediated receptor internalization and down-regulation (15), our next question was whether the Y1045F mutant exposed to EGF would show a similar phenotype. Recent publications had suggested some discrepancies regarding Y1045F internalization (5–7) as described under “Discussion.” To quantify the amount of the EGFR remaining at the plasma membrane after treatments, a reverse ubiquitination experiment was designed, using the principles of the degradation assay described above. Briefly, CHO cells co-transfected with c-Cbl and either the WT EGFR or with the Y1045F mutant were treated with EGF or H2O2 for the indicated time points, and then biotinylation of the surface receptors carried out. The EGFR levels were determined after cell lysis and immunoprecipitation. As shown in Fig. 2A, fewer WT receptors treated with EGF remained available at the plasma membrane for biotinylation compared with the Y1045F mutant following EGF treatment or WT EGFR treated with H2O2. However, a slow decline in Y1045F receptor could also be observed over time, similar to the levels seen upon treatment with the WT receptor with H2O2, suggesting that this mutant receptor was still being internalized as revealed by confocal analysis (Fig. 2C). Yet, whereas a portion of the WT EGFR also disappeared from the plasma membrane under H2O2, only the receptors treated with EGF (both WT and Y1045F mutant) are co-localized with EEA1, an early endosomal marker (Fig. 2, B and C).

As shown (Fig. 2A), the majority of EGFR ubiquitination was eliminated using the Y1045F mutant with no change in total ubiquitinylation (Fig. 1C). To confirm that any basal remaining level of ubiquitinylation was not due to c-Cbl ubiquitin ligase activity, we transfected CHO cells with the WT EGFR and either WT c-Cbl or a dominant negative c-Cbl (c-Cbl 381A) (21). We found that, similar to the Y1045F mutant, the dominant negative c-Cbl-transfected CHO cells still showed EGFR internalization (Fig. 3D) despite the deficiency of c-Cbl-
mediated receptor ubiquitinylation (Fig. 3A). These data are in agreement with Jiang and Sorkin’s biochemical internalization studies of c-Cbl 381A-transfected HeLa cells (5), which demonstrate slower but definite EGFR internalization with c-Cbl 381A when compared with cells transfected with WT c-Cbl. Transfection with the dominant negative c-Cbl had no effect on total ubiquitinylation (Fig. 3B) but did result in prolonged downstream signaling of AKT upon EGF treatment (Fig. 3C), similar to cells transfected with the Y1045F mutant (Fig. 1B). Furthermore, confocal microscopy showed clear co-localization of both the Y1045F receptor with an early endosome marker (EEA1) (Fig. 2C) as well as the WT EGFR transfected with c-Cbl 381A (Fig. 3D), suggesting that not only were the receptors deficient in c-Cbl-mediated ubiquitinylation internalized, but that they were also able to reach the early endosomes. Therefore, it can be concluded from these data that c-Cbl-mediated ubiquitinylation of the EGFR is not needed for receptor internalization but is required for later events leading to degradation of the receptor.

**Tyr-1045 Mutation but Not H2O2 Exposure Allows c-Cbl Binding to EGFR**—To investigate the mechanism of EGFR resistance to degradation under oxidative stress and to explore further whether it is solely dependent on c-Cbl association with Tyr-1045, we compared the ability of the mutant and the wild type receptor to bind c-Cbl. Despite its inability to be phosphorylated at Tyr-1045, the Y1045F mutant was still associated with c-Cbl as shown in Figs. 1A and 4. Previously, we demonstrated that H2O2 exposure does not enhance Tyr-1045 phosphorylation and association between EGFR and c-Cbl (15), and as expected Fig. 4 shows that, upon H2O2 treatment, there was no EGFR co-localization with c-Cbl. We considered that the difference may be due to the ability of c-Cbl to bind EGFR not only via Tyr-1045, but also via the Grb2 adaptor (22). Indeed, through immunoprecipitation analysis we found that, under exposure to H2O2, although c-Cbl binds Grb2, the latter failed to associate with EGFR (Fig. 5).

Therefore, under H2O2 exposure, no c-Cbl binding to the receptor could be observed, whereas the Y1045F mutant could still be slowly associated with c-Cbl (see Fig. 1: binding of c-Cbl to the Y1045F mutant was observed only after 1 h of EGF treatment). But, in both cases, no c-Cbl-mediated EGFR ubiquitinylation could be observed (Fig. 2A). Despite c-Cbl association with the Y1045F mutant (probably via Grb2), the receptor was not ubiquitinylated (Fig. 2A), suggesting that, when bound only via Grb2, c-Cbl could not function as an E3 ligase of the EGFR, but may still serve as an adaptor protein for EGFR internalization.

**Activated c-Cbl Is Not Required for EGFR Entry into the Early Endosomes**—Because under oxidative stress much of the EGFR remained localized at the plasma membrane and failed to co-localize with an early endosomal marker (Fig. 2B), it raised the question of whether c-Cbl might have a role not only
as an E3 ligase, but also as an adaptor, which is essential for entry into the clathrin-dependent pathway through the early endosomes and is deficient under oxidative stress.

The first question was whether c-Cbl had to be activated to allow internalization of EGFR, or if it simply could act as an adaptor protein, leading to internalization. For this purpose, we have utilized the Src kinase inhibitor, PP1, that was previously shown to inhibit c-Cbl phosphorylation as well as its E3 ligase activity in T47D cells (Kassenbrock et al. (23)). Human lung epithelial A549 cells, which are natural EGFR expressers and also express high levels of c-Src, were treated with EGF alone or in the presence of various concentrations of PP1, and the effects of PP1 on c-Cbl-mediated receptor down-regulation were determined (Figs. 6 and 7). Fig. 6A shows that 5 μM PP1 was sufficient to block both basal and EGF-induced c-Src phosphorylation. Yet, as shown in Fig. 6B (and also by Kassenbrock...
et al. (23)) the inhibitory effects of PP1 on c-Cbl phosphorylation and E3 ligase activity only appear at higher concentrations of the inhibitor.

Because PP1 concentrations required for inhibition of Src family kinases are considerably lower than 50 μM (23) (Fig. 6A), it is postulated that c-Cbl inhibition by the higher PP1 concentration reflects a nonspecific inhibitory effect on another tyrosine kinase (23). The data in Fig. 6B, however, indicate no significant effects of either the low or high PP1 concentrations on total tyrosine phosphorylation of the EGFR, suggesting that PP1 does not affect the intrinsic tyrosine kinase activity of the EGFR. Similarly, no inhibitory effects of PP1 on Tyr-1045 phosphorylation or on c-Cbl binding to the EGFR could be observed, suggesting that pharmacologically inactive c-Cbl can still bind to the receptor. The high correlation between the inhibition of c-Cbl phosphorylation and EGFR ubiquitinylation suggests that high concentrations of PP1 can abrogate c-Cbl phosphorylation and its E3 ligase activity and, thus, EGFR ubiquitinylation in A549 cells.

Confocal analysis was next used to study EGFR internalization in the presence of PP1, demonstrating that PP1 did not significantly inhibit EGFR internalization (Fig. 6C). To localize the EGFR with respect to endosomes, we used antibodies against the early endosomal marker EEA1. Cells were first treated on ice with EGF conjugated to Alexa Fluor 488 (AF488-EGF) in the absence or presence of 50 μM PP1 and then were incubated at 37 °C for 30 min. Fig. 6C demonstrates that in A549 cells EGF-bound receptor staining was restricted to the plasma membrane when cells were incubated on ice. Following incubation at 37 °C the AF488-EGF was rapidly accumulated in cytoplasmic vesicles that co-localized with EEA1 (Fig. 6C, merged panel). Incubations with PP1 did not affect EGFR ac-
ubiquitinylation may be necessary for EGFR exit from the early endosomes and, thus, its eventual trafficking to the lysosome, but are not necessary for EGFR internalization.

**DISCUSSION**

We have previously shown that EGFR phosphorylation and downstream signaling are targeted by oxidative stress (14, 15). Briefly, our initial studies demonstrated that exposure of cells to H$_2$O$_2$-mediated oxidative stress enhances tyrosine phosphorylation of the EGFR without being accompanied by c-Cbl-mediated receptor internalization and degradation. Our findings led us to propose that the abrogated phosphorylation of Tyr-1045, but not other Tyr residues of the EGFR, is the basis for aberrant receptor down-regulation under oxidative stress (15).

In the present study we proceeded to examine this hypothesis and proposed that a Y1045F mutation of the EGFR would mimic the phenotype of the EGFR under oxidative stress by allowing prolonged EGFR signaling at the plasma membrane. We examined the role of Tyr-1045 in EGFR down-regulation using CHO cells ectopically expressing Y1045F mutant EGFR and c-Cbl and observed that, similar to our previous studies with H$_2$O$_2$, treatment of Y1045F EGFR with EGF conferred prolonged receptor tyrosine phosphorylation, which resulted in prolonged downstream signaling. Furthermore, by using a protein biotinylation assay, we demonstrated that activation of Y1045F EGFR by EGF was not followed by receptor degradation. These results are in agreement with previous publications that examined the role of Tyr-1045 in EGFR down-regulation (5, 8, 24).

Through use of the Y1045F mutant EGFR model system, we have identified that c-Cbl binding does not solely depend on Tyr-1045 phosphorylation, and thus the deficiency of c-Cbl binding under oxidative stress cannot be simply attributed to abrogation at this phosphorylation site. Indeed, recent studies suggested that c-Cbl is recruited to the activated EGFR through both direct and indirect binding (5, 24). Whereas direct c-Cbl-EGFR interaction is mediated through phosphorylated Tyr-1045 on EGFR (8), indirect c-Cbl-EGFR interaction is primarily mediated through Grb2. The SH3 domain of Grb2 binds to proline-rich sequences of c-Cbl, whereas the SH2 domain binds to autophosphorylated EGFR (26, 27). Interestingly, the two sites for Grb2 recruitment to the EGFR, Tyr-1068 and Tyr-1086, are phosphorylated under H$_2$O$_2$ exposure (albeit at lower levels than with EGF), but Grb2, which is still bound to c-Cbl, does not bind the EGFR (Fig. 5). Furthermore, Shc, whose phosphorylation and binding to Grb2 has been shown to be responsible for Grb2 recruitment to the EGFR (27), is still phosphorylated under H$_2$O$_2$ exposure and is still able to bind to the EGFR (data not shown). Therefore, it is possible that Grb2 fails to bind to the EGFR due to insufficient phosphorylation of Tyr-1068 and Tyr-1086, or due to conformational changes in the EGFR, two avenues which are currently under investigation in our laboratory. Recent studies by Jiang et al. (22), demonstrated that double Tyr $\rightarrow$ Phe mutations of the EGFR at Grb2 binding sites abolishes receptor internalization upon ligand induction. Whether or not these mutations interfere with c-Cbl binding to the EGFR is yet to be determined. Thus, our studies with the Y1045F mutant have led to a better understanding of why c-Cbl fails to bind to the EGFR under oxidative stress. This is due to both the abrogation of phosphorylation of Tyr-1045 and the lack of Grb2 binding, which results in a receptor unable to undergo normal EGFR internalization through the early endosomes and down-regulation (Fig. 8).

Additionally, our studies provide novel and detailed data regarding the role of c-Cbl in EGFR signaling and trafficking. Although extensively investigated, the role of c-Cbl in EGFR

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**FIG. 5.** Grb2 fails to associate with both WT-EGFR and Y1045F mutant under H$_2$O$_2$, but remains associated with c-Cbl. CHO cells stably transfected with either c-Cbl and WT-EGFR or c-Cbl and the Y1045F mutant were serum-starved overnight, followed by incubation with 100 ng/ml EGF or 1 unit/ml GO for 30 min. The cells were then lysed, and EGFR or c-Cbl was immunoprecipitated (IP) from cell lysates using anti-EGFR C225 antibody or anti-c-Cbl, C-15, antibody, respectively. Precipitated proteins were separated by SDS-PAGE and immunoblotted with anti-Grb2, C-25 antibody, or anti-EGFR, RR2 antibody, as indicated. Results are representative of at least three independent experiments.
down-regulation is still contradictory. The initial findings by Levkovitz et al. (8), that binding of c-Cbl to the EGFR upon ligand induction promotes receptor ubiquitinylation, is now well established. However, the dual role for c-Cbl in EGFR degradation as well as its role in recruiting the EGFR to coated pits through its interaction with cin85 and the endophilin complex (28) have led to some controversy over when and where the ubiquitin ligase activity of c-Cbl is required. Both the E3 ligase activity of c-Cbl and its function as an adaptor protein have been suggested as being essential for efficient receptor internalization (5). Recent publications have also suggested some discrepancies regarding Y1045F internalization (5–7). Jiang and Sorkin (5) showed data suggesting that the Y1045F mutant was internalized despite its inability to undergo ubiquitylation, whereas Mosesson et al. (6) demonstrated that the Y1045F mutant is internalization-resistant. Additionally, Duan et al. (7) showed that in mouse embryonic fibroblast Cbl−/− cells, the EGFR could still be internalized. These studies have led to some confusion about when and where the ubiquitin ligase activity of c-Cbl is required. The significance of the c-Cbl requirement and its mediation of ubiquitinylation for EGFR exit from the early endosomes may be very important and thus merits future studies. Several proteins with ubiquitin interaction domains have been shown to be necessary for EGFR transfer from the early endosomes to other vesicular bodies for degradation (25, 31). According to these studies, these proteins, Hrs and Tag 101, seem to be involved in a large sorting complex that is "somehow" respon-
sible for coupling EGFR transfer between these two vesicular compartments. Although the mechanism has yet to be identified, one can imagine a situation where this large protein sorting complex binds to the ubiquitylated EGFR, thus allowing its transfer into the late endosomes.

To block receptor ubiquitylation, we utilized an inhibitor of Src family kinases, PP1 (Fig. 6B). Administration of PP1 to A549 cells is shown to block EGF-induced phosphorylation of c-Cbl (but not of the EGFR) and the ubiquitination of the EGFR, which is in agreement with the findings of Kassenbrock et al. in T47D cells (23). We observed efficient binding of c-Cbl to the EGFR in the presence of PP1, in both wild type and Y1045F receptors, and our findings imply that the tyrosine phosphorylation of c-Cbl is not required for its efficient binding to the EGFR or for its role in EGFR entry into the early endosomes. In addition, we have shown that, similar to the experiments with the Y1045F mutant in CHO cells, EGF induction in the presence of PP1 enhances the internalization of non-ubiquitylated receptors, although PP1-treated cells showed a slightly slower internalization rate, as demonstrated by residual fluorescent EGF staining at the plasma membrane after 30 min of incubation. However, at 45 min, EGFR was internalized into early endosomes (Fig. 6B). Thus, PP1 treatment abolished EGF-induced c-Cbl phosphorylation and EGFR ubiquitylation but not internalization. The data also imply that c-Cbl binding to the EGFR does not require tyrosine phosphorylation of c-Cbl or the activity of Src family kinases. Furthermore, we have utilized this inhibitor to identify where c-Cbl-mediated ubiquitylation is required in EGFR trafficking. At extended time points EGFR treated with 50 μM PP1 and

**Fig. 7.** c-Cbl-mediated ubiquitylation is required for EGFR exit from the early endosomes. A, serum-starved A549 cells were preincubated with 50 μM PP1 for 40 min at 37 °C and then treated with 100 ng/ml EGF for 45 min to allow EGFR internalization. At 45 min, cells were washed three times with PBS and replaced with media containing only EGF for the indicated time points. Cells were lysed, and immunoprecipitation of the EGFR was carried out. Analysis by SDS-PAGE was followed by immunoblotting using anti-EGFR RK2 antibody, anti-phosphotyrosine PY20, and anti-c-Cbl C-15 antibodies. B, serum-starved A549 cells were preincubated for 40 min at 37 °C with 100 μM monensin and treated and analyzed as described in part A. C, serum-starved A549 cells grown on coverslips were treated as described above (using EGF-Texas Red) for the indicated time points. Cells were fixed, incubated with primary antibody anti-EEA1, and secondary antibody Alexa Flour 488. Co-localization was performed by confocal microscopy. Both confocal and Western data results are representative of two independent experiments.
role of c-Cbl in EGFR down-regulation to gain a better understanding of how a deficiency in c-Cbl binding could lead to tumorigenesis under oxidative stress. Our major findings (Fig. 8) are that c-Cbl binding to the EGFR is sufficient to enhance receptor internalization, whereas the E3 ligase activity of c-Cbl and EGFR ubiquitylation are required for EGFR trafficking out of the early endosomes and eventual transport to the lysosome for efficient degradation of the receptor. We have successfully utilized the Src family inhibitor PP1 to block c-Cbl phosphorylation and E3 ligase activity without affecting the intrinsic kinase activity of the EGFR. This allowed us to extend our studies in ectopically expressed CHO cells to a more physiological cell system such as the A549 airway epithelial cells and provided us with more versatility by enabling restoration of the ubiquitylated state of the EGFR. Finally, our study implies that the lack of Tyr-1045 phosphorylation during exposure to oxidative stress is probably not the only factor in the inability of EGFR to enter the early endosomes. Rather, the role of Grb2 in the recruitment of c-Cbl as an adaptor adds an additional dimension to the function of the EGFR under oxidative stress and is currently under investigation.

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FIG. 8. Schematic model for the role of c-Cbl in EGFR trafficking. In the presence of EGF, the WT-EGFR recruits c-Cbl both via the Tyr-1045 site and indirectly via Grb2. Inactive c-Cbl (not as an E3 ligase) is sufficient for EGFR internalization through the early endosomes. Exit from the early endosomes requires that the EGFR be ubiquitylated by c-Cbl. On the other hand, under oxidative stress the EGFR fails to enter c-Cbl entirely (both indirectly via Grb2 and via Tyr-1045). Consequently, the EGFR under O2 exposure fails to enter the early endosomes, leading to an activated receptor unable to undergo normal down-regulation, which may lead to tumorigenesis.

EGF remain associated with EEA1, an early endosomal marker (Fig. 6C). Only when 50 μM PP1 was removed, and c-Cbl phosphorylation and EGFR ubiquitylation recovered, did EGFR migrate out of the early endosomes.

There are several benefits for using PP1 as an inhibitor of EGFR ubiquitylation. Not only can we remove the inhibitor and regain EGFR ubiquitylation, but we can also avoid using ectopic expression. Ectopic expressions of c-Cbl and EGFR, intact or mutated, are widely used in studies of receptor trafficking. The inherent problems in the interpretation of these data involve the potential multiple effects of these non-physiological manipulations on the inserted molecules and on the downstream signaling pathways. For example, overexpression of the EGFR may change the stoichiometric balance between components of the endocytic machinery, which may disrupt down-regulation of cell surface receptors (22). Mutagenesis, on the other hand, can cause conformational changes in sites of the protein that are distal to the site of mutation. Thus, the interpretations of these experiments have to be cautiously considered, and the results should be validated in more “natural” cell systems. Because A549 human airway epithelial cells were previously shown to be EGFR expressers, and because the EGFRs in these cells respond to EGF with desensitization mechanisms, including c-Cbl-mediated ubiquitylation, internalization, and degradation of the receptor, we further utilized these cells to study endogenous EGFR internalization in the absence of ubiquitylation (Fig. 7).

In summary, in the present study we further examined the
