Essential Role of c-Cbl in Amphiregulin-Induced Recycling and Signaling of the Endogenous Epidermal Growth Factor Receptor†

Aleksander Baldys,[‡,§] Monika Göoz,[‡,§] Thomas A. Morinelli,[‡,§] Mi-Hye Lee,[§,||] John R. Raymond, Jr.,[‡] Louis M. Luttrell,[§,||] and John R. Raymond, Sr.*[,‡,§]

Nephrology and Endocrinology Divisions, Department of Medicine, Medical University of South Carolina, and Medical and Research Services, Ralph H. Johnson Veterans Affairs Medical Center, Charleston, South Carolina 29425

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ABSTRACT: The intracellular processing of the epidermal growth factor receptor (EGFR) induced by epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) has been studied meticulously, with the former resulting in EGFR degradation and the latter in EGFR recycling to the plasma membrane. However, little is known about how other EGF family growth factors affect the trafficking of the EGFR. Additionally, although both EGF and TGF-α have been shown to effectively induce initial c-Cbl (ubiquitin ligase)-mediated ubiquitination of the EGFR, limited information is available regarding the role of c-Cbl in the trafficking and signaling of recycling EGFR. Thus, in this study, we investigated the roles of c-Cbl in endogenous EGFR trafficking and signaling after stimulation with amphiregulin (AR). We demonstrated that a physiological concentration of AR induced recycling of the endogenous EGFR to the plasma membrane, which correlated closely with transient association of the EGFR with c-Cbl and transient EGFR ubiquitination. Most importantly, we used c-Cbl small interfering RNA (siRNA) duplexes and a c-Cbl dominant negative mutant to show that c-Cbl is critical for the efficient transition of the EGFR from early endosomes to a recycling pathway and that c-Cbl regulates the duration of extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase (ERK1/2 MAPK) phosphorylation. These data support novel functions of c-Cbl in mediating recycling of EGF receptors to the plasma membrane, as well as in mediating the duration of activation (transient vs sustained) of ERK1/2 MAPK phosphorylation.

The epidermal growth factor receptor (EGFR)[1] belongs to a family of cell surface receptor tyrosine kinases, which includes four ErbB members, i.e., EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4 (reviewed in ref *1*). Many different growth factors can serve as ligands for the EGFR, and these include epidermal growth factor (EGF), transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), amphiregulin (AR), epiregulin (EPR), and epigen (EPG). All EGFR ligands are synthesized as membrane proteins and are subsequently released from the cell surface by regulated proteolysis. However, little is known about what dictates the cleavage and shedding of different EGFR ligands or, most importantly, about the physiological and pathological relevance of the different cognate ligands. To date, how different EGFR ligands could serve distinct functions despite their shared interactions with the same receptor remains an enigma.

Ligand binding to the EGFRs causes the formation of homo- and heterodimers, a process that subsequently induces autophosphorylation through activation of the EGFR tyrosine kinase activity. Following activation, the EGFR undergoes internalization and endocytic trafficking. After endocytosis, some receptors recycle from endosomes back to the plasma membrane, whereas others enter the degradative pathway to late endosomes and lysosomes, a process that results in receptor downregulation. In that regard, it is well established that EGF, but not TGF-α, triggers efficient degradation of the EGF receptors (*2, 3*). A recent report (*4*) also demonstrated that AR does not induce significant EGFR degradation.

There have been significant advances in the understanding of how receptor trafficking and signaling are functionally interrelated (*5*), yet this relationship still remains obscure. The signaling of the activated EGFR involves numerous downstream pathways, including mitogen-activated protein kinases, phosphatidylinositol 3-kinase, c-Src, and phospholipase C γ/protein kinase C. These complex signal trans-

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* To whom correspondence should be addressed: Room 213, Colcock Hall, 179 Ashley Ave., Charleston, SC 29425. Phone: (843) 792-3031. Fax: (843) 792-5110. E-mail: raymondj@musc.edu.

‡ Nephrology Division, Department of Medicine, Medical University of South Carolina.

§ Ralph H. Johnson Veterans Affairs Medical Center.

|| Endocrinology, Department of Medicine, Medical University of South Carolina.

[1] Abbreviations: AR, amphiregulin; BTC, betacellulin; EEA1, early endosome antigen 1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EPG, epigen; EPR, epiregulin; ERK1/2 MAPK, extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase; HEK293, human embryonic kidney 293; HB-EGF, heparin-bound EGF-like growth factor; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; LAMP, lysosome-associated membrane protein; SE, standard error; TGF-α, transforming growth factor-α.

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duction cascades modulate cell proliferation, differentiation, adhesion, migration, survival, and death. Whereas EGFR signaling is crucial for many normal cellular processes, aberrant EGFR activation has been implicated in the pathophysiology of hyperproliferative diseases such as cancer.

The mammalian Cbl proteins constitute a highly conserved family of three ubiquitin ligases, known as c-Cbl, Cbl-b, and Cbl-c (reviewed in ref *6*). In recent years, Cbl has emerged as a critical player in regulating EGFR endocytic trafficking (*7, 8*). Numerous studies have provided direct evidence of the role of EGF-induced, Cbl-mediated, sustained EGFR ubiquitination in receptor targeting to lysosomes (*3, 9*−*15*). Importantly, although TGF-α has been shown to induce transient association of the EGFR with Cbl and receptor ubiquitination (*3*), there have been no reports so far in the literature addressing possible roles of Cbl in receptor recycling.

The role of c-Cbl as a regulator of signal transduction, and consequently cell function and development, is now well-established (*16*). Evidence suggests that dysregulation and/or disruption of the function of c-Cbl contributes to the development of many pathological conditions, including immunological and malignant diseases. The role of c-Cbl in signaling is thought to be based largely on its ubiquitin ligase activity, but many cellular events are dependent on its function as an adaptor molecule (*16*).

Others previously have shown that EGF and TGF-α induce differential fates of the internalized EGFR, with the former resulting in EGFR degradation and the latter in EGFR recycling. However, although c-Cbl has been implicated in the regulation of EGFR degradation, possible roles for c-Cbl in EGFR recycling have not yet been addressed. Therefore, in this study, we examine the roles of c-Cbl in ligand-specific EGFR trafficking and signaling. Concentrating on two members of the EGFR ligand family, i.e., EGF and AR, we show that AR and EGF induced similar patterns of short-term EGFR and c-Cbl phosphorylation, physical association of c-Cbl with the EGFR, and EGFR ubiquitination; however, as previously reported for TGF-α (*3*), the effects of AR are much more transient than those of EGF. Most importantly, our new data implicate c-Cbl in the active sorting of the EGFR to recycling endosomes. We also show that c-Cbl regulates the duration of AR-induced extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase (ERK1/2 MAPK) activation. Taken together, our results shed some light on the new, unexplored aspects of specialized endocytic sorting to recycling pathways.

EXPERIMENTAL PROCEDURES

Materials. Human embryonic kidney (HEK293) cells were purchased from American Type Culture Collection (Manassas, VA). Human recombinant EGF, human recombinant AR, chloroquine, monensin, and all other biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA). Sheep polyclonal anti-EGFR and anti-phosphotyrosine (clone 4G10) antibodies were from Upstate Millipore (Billerica, MA). Rabbit polyclonal anti-Rab11 was from Zymed Laboratories (South San Francisco, CA). Mouse monoclonal anti-early endosome antigen 1 (EEA1) (clone 14) and mouse monoclonal anti-c-Cbl (clone 17) were from BD Transduction Laboratories (Franklin Lakes, NJ). Mouse monoclonal anti-lysosome-associated membrane protein (LAMP) (clone H4A3) was from BD Pharmingen (San Diego, CA). Mouse monoclonal anti-Cbl-b (clone G-1) was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-ubiquitin (clone FK2) was from Biomol (Plymouth Meeting, PA). Rabbit anti-phospho-ERK1/2, mouse anti-ERK1/2, and rabbit anti-phospho-EGFR (Tyr-1173) antibodies were from Cell Signaling Technologies (Danvers, MA). Mouse monoclonal anti-β-actin (clone AC-15) antibody was from Sigma-Aldrich. Peroxidase-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA) and Rockland Immunochemicals (Gilbertsville, PA). SDS−PAGE molecular mass markers were from Bio-Rad (Hercules, CA).

Cell Culture, RNA Interference Experiments, and DNA Transfection. HEK293 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen Gibco, Carlsbad, CA) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were grown to ∼75% confluence following which they were placed in serum-deprived binding medium (MEM supplemented with 0.1% bovine serum albumin) for 24 h prior to treatments. For synchronized ligand pulse experiments, cells at 4 °C were incubated with agonists for 45 min in serum-deprived binding medium containing 20 mM HEPES (pH 7.4). Then, the cells were rinsed with ice-cold PBS to remove unbound ligand, following which the bound ligand stimulated the EGFR upon exposure to pre-warmed ligand-free medium at 37 °C.

A mixture of four SMARTselection-designed siRNAs targeting one gene (Thermo Fisher Scientific Dharmacon, Inc., Lafayette, CO) was transfected using oligofectamine (Invitrogen) reagent according to the manufacturer's instructions. A pool of four siGenome nontargeting siRNAs, designated as scrambled (SCR) siRNA, was used as a control; 72 h following transfection, cell lysates were assayed for silencing effectiveness by Western blotting and immunofluorescence staining.

The expression constructs pcDNA3GFP-Cbl-WT and pcDNA3GFP-Cbl-N have been described previously and were kindly provided by H. Band (*17*). HEK293 cells were transiently transfected with the constructs described above using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

Western Blotting. After treatments, cells were rinsed briefly with PBS and extracted with RIPA buffer (150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% NP-40, and 0.1% SDS in PBS) containing protease inhibitors. Cells subsequently were sonicated, and protein concentrations were determined by the BCA assay (Pierce, Rockford, IL). Equal amounts of proteins were separated by SDS−PAGE on 4 to 12% polyacrylamide gels (Invitrogen), transferred to PVDF membranes, and blocked with 5% milk in PBS for 1 h at room temperature. Following several washes with PBS containing 0.1% Tween, the membranes were incubated with the appropriate dilutions of primary and peroxidase-conjugated secondary antibodies (as directed by the manufacturer) in blocking solution. Immunoblotted proteins were detected using ECL reagents (GE Healthcare Amersham Biosciences, Piscataway, NJ).

Biotinylation of Cell Surface Proteins. HEK293 cells grown on 100 mm dishes were washed one time with ice-cold PBS and incubated with 0.5 mg/mL sulfo-NHS-biotin (Pierce) for 30 min at 4 °C to label surface proteins. Cells then were washed with 15 mM glycine to quench excess, unreacted biotin. After the indicated treatments, cells were rinsed briefly with ice-cold PBS and extracted with Triton lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and 5 mM EDTA] supplemented with protease inhibitor cocktail III (EMD Calbiochem, San Diego, CA), 1 mM PMSF, and phosphatase inhibitors [HALT phosphatase inhibitor cocktail (Pierce)]. Equal amounts of proteins (0.5 mg) were precleared by incubation for 30 min at 4 °C with 30 μL of protein A/G Agarose beads (Santa Cruz Biotechnology). After a brief centrifugation, the supernatants were removed and incubated overnight at 4 °C with 50 μL of streptavidin−agarose beads (Novagen, Madison, WI). The samples then were centrifuged and washed three times with 1 mL of Triton lysis buffer. Proteins were eluted from the beads using Laemmli sample buffer. Samples subsequently were analyzed by SDS−PAGE and Western blotting.

Immunoprecipitation. After the indicated treatments, HEK293 cells grown in 150 mm dishes were scraped into ice-cold PBS and centrifuged at 200*g*. Pellets were lysed in 1 mL of Triton lysis buffer supplemented with protease and phosphatase inhibitors, as described above. Equal amounts of proteins (1.5 mg) were precleared by incubation for 30 min at 4 °C with 30 μL of protein A/G Sepharose beads. After a brief centrifugation, the supernatants were removed and incubated overnight at 4 °C with either 8 μg of anti-EGFR or 13 μg of anti-Cbl antibodies. Immunoprecipitates were captured with 50 μL of protein A/G beads at 4 °C for 1 h. The samples were then centrifuged and washed three times with 1 mL of Triton lysis buffer. Proteins were eluted from the beads using Laemmli sample buffer. Samples were subsequently analyzed by SDS−PAGE and Western blotting.

Immunofluorescence Staining and Confocal Microscopy. Cells were grown on 35 mm lysine-coated, glass-bottom culture dishes (MatTek Corp., Ashland, MA). After treatments, cells were fixed with freshly prepared 3.7% paraformaldehyde in PBS for 15 min at room temperature. Subsequently, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 5 min, following which nonspecific binding sites were blocked with 3% normal serum (Santa Cruz Biotechnology) in PBS for 1 h. Incubations with the appropriate dilutions of primary and Alexa Fluor-conjugated secondary antibodies (as directed by the manufacturer) were performed in blocking solution. Confocal microscopy was performed using a Zeiss LSM 510 META laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a 60× objective, using the following laser wavelengths: excitation at 488 nm and emission at 505−530 nm; excitation at 543 nm and emission at 560−615 nm. Quantifications of the colocalization coefficients, derived from measured pixel overlaps between the EGFR and EEA1, Rab11, and LAMP, were performed using Zeiss LSM 510 colocalization analysis software. The mean values were averaged from at least three independent single-cell images.

Receptor Recycling Assay. HEK293 cells grown in six-well plates were incubated with 100 ng/mL AR at 4 °C for 45 min in serum-deprived binding medium containing 20 mM HEPES (pH 7.4). Then, the cells were rinsed with ice-cold PBS to remove unbound ligand, following which cells were incubated for 0, 15, 30, and 60 min in prewarmed ligand-free medium at 37 °C. The cells were then placed on ice and rinsed twice with ice-cold PBS, followed by a 7 min incubation with a low-pH stripping buffer [150 mM acetic acid (pH 2.7) containing 150 mM NaCl] to remove surface-bound ligands. The numbers of available cell surface ligand-binding sites were determined by incubating cells for 90 min on ice with [^{125}I]EGF (PerkinElmer, Boston, MA), as previously described (*18*). The specific activity of [^{125}I]EGF was approximately 5×10^5 cpm/ng. The cells then were washed three times with ice-cold PBS and solubilized with 1 mL of 0.1 N NaOH containing 0.1% SDS, and the bound radioactivity was measured in a gamma counter. Nonspecific binding was assessed in the presence of excess unlabeled HB-EGF.

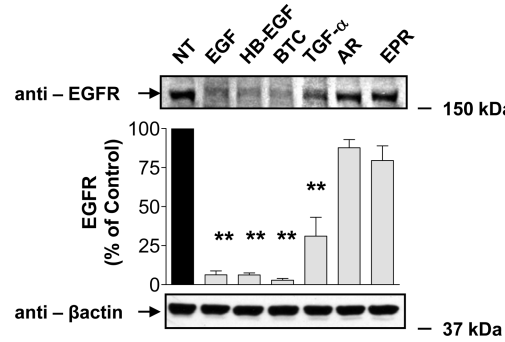

FIGURE 1: Differential fates of the EGFR induced by EGFR ligands. Serum-deprived HEK293 cells were treated at 37 °C with vehicle (NT), 100 ng/mL EGF, HB-EGF, BTC, TGF-α, AR, or EPR for 180 min, extracted with RIPA buffer, and subsequently immunoblotted with anti-EGFR and anti-β-actin antibodies. Data shown are representative of three independent experiments. Results are means ± SE ($n = 3$). Asterisks indicate a p of <0.01 vs vehicle (NT).

Statistical Analyses. Statistical significance was determined using a paired two-tailed t test and analysis of variance (ANOVA) with Dunnett's or Bonferroni post-test to correct for multiple comparisons (GraphPad Prism, version 4). p values of <0.05 were considered to be statistically significant.

RESULTS

Differential Fates of the EGFR Induced by EGFR Ligands. EGFR intracellular trafficking has been intensely studied, yet the overwhelming majority of studies has focused primarily on one ligand, EGF. Although the effects of some other EGFR ligands on the EGFR also have been investigated (*3, 4, 18, 19*), little is known about the effects of most of those other cognate EGFR ligands. Hence, we examined the effects of six known EGFR ligands, i.e., EGF, HB-EGF, BTC, TGF-α, AR, and EPR, on HEK293 cells. We analyzed the fate of the EGFR by Western blotting following treatment with a saturating concentration (100 ng/mL) of individual ligands for 180 min. As seen in Figure 1, stimulation with HB-EGF or BTC resulted in a degradation of the EGFR similar in magnitude to that caused by EGF. In contrast, the level of degradation in response to TGF-α treatment was significantly reduced as compared with that with EGF. AR- or EPR-stimulated cells did not show any significant loss of EGFR immunoreactivity. Because saturating concentrations of AR and EPR do not induce degradation of EGFR, we

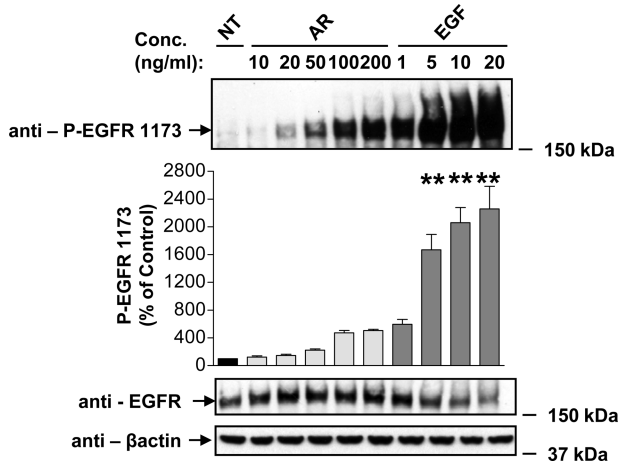

FIGURE 2: Effects of AR and EGF on EGFR autophosphorylation. Serum-deprived HEK293 cells were treated at 37 °C with vehicle (NT), 10, 20, 50, 100, or 200 ng/mL AR, or 1, 5, 10, or 20 ng/mL EGF for 2 min. After being washed with ice-cold PBS, cells were extracted with RIPA buffer, and cell lysates were immunoblotted with anti-phospho-EGFR Tyr-1173 antibody. Blots were then stripped and reprobed for the total amount of EGFR and β-actin to normalize for loading. Data shown are representative of three independent experiments. Results are means ± SE ($n = 3$). Asterisks indicate a p of <0.01 vs vehicle (NT).

reasoned that these ligands might induce recycling of the EGFR. To address the possibility that c-Cbl affects trafficking and signaling of recycling EGFRs, we chose to compare for the rest of our studies the effects of AR with those of well-characterized EGF.

Effects of AR and EGF on EGFR Autophosphorylation. Because AR has a lower affinity for the EGFR than does EGF (*20*), we varied the concentrations of EGF and AR to find optimal concentrations that would bring about similar extents of short-term EGFR phosphorylation. This approach enabled us to characterize trafficking of EGFRs that were autophosphorylated and/or activated to a similar degree by the two ligands. We examined the effects of various concentrations of AR and EGF on phosphorylation of the EGFR at Tyr-845 (c-Src-mediated), as well as Tyr-1068 and Tyr-1173 (major autophosphorylation sites). Phosphorylation of Tyr-845 is believed to stabilize the receptor activation loop and is required for the mitogenic function of the receptor. Phosphorylated Tyr-1068 is involved in receptor trafficking, whereas phosphorylated Tyr-1173 plays an important role in MAPK activation. Our concentration−response studies demonstrated that treatment of HEK293 cells with physiological concentrations of ligand, 100 ng/mL (9 nM) AR or 1 ng/mL EGF (0.17 nM), for 2 min caused comparable levels of phosphorylation of Tyr-1173 (Figure 2). We had similar findings for EGFR Tyr-845 and Tyr-1068 (data not shown). Accordingly, we used 100 ng/mL AR or 1 ng/mL EGF for the remainder of our studies.

Differential Trafficking of the EGFR Induced by EGF and AR. To further examine the intracellular localization of the AR- or EGF-treated EGFR, we performed experiments in the presence or absence of chloroquine, which blocks lysosomal degradation, or monensin, which interferes with protein recycling (*21*). To circumvent a low level of endogenous EGFR expression and, most importantly, the effects of simultaneous EGFR processing, i.e., receptor endocytosis, sorting, recycling, and degradation, we used a widely employed synchronization approach (*3, 13, 22*−*25*). Briefly, following prebinding of the ligand at 4 °C, the cells were washed to remove unbound ligand and then shifted back to 37 °C, which enabled initiation of synchronous, ligand-induced EGFR processing. We employed various markers of subcellular organelles, i.e., EEA1 for early endosomes, Rab11 for the perinuclear slow recycling compartment, and LAMP for lysosomes (Figure 3). Five minutes following rewarming of the cells pretreated with either AR or EGF, EGFRs extensively colocalized with EEA1, suggesting that EGFRs enter early endosomes after stimulation with either AR or EGF (Figure 3A, 5 min). In contrast, after 15 min, the AR-stimulated EGFRs partially localized to both early (EEA1-positive) and recycling (Rab11-positive) endosomes, whereas EGF-activated EGFRs only partially colocalized with EEA1 and did not appear to colocalize with Rab11 (Figure 3B, 15 min). Thirty minutes after being rewarmed, the AR-stimulated EGFRs appeared predominantly on the plasma membrane; the EGFRs were fully recycled after 60 min. On the other hand, the paucity of staining for EGFRs treated with EGF after being rewarmed for 30 and 60 min was indicative of EGFR degradation (Figure 3C). Importantly, our studies in the presence of monensin and chloroquine demonstrated clear, predominant colocalization of AR-stimulated EGFRs in Rab11-positive vesicles and EGF-stimulated EGFR in LAMP-positive lysosomes, respectively (Figure 3D; AR, 60 min/M; EGF, 60 min/Ch). It should be noted that consistent with previous studies (*21, 26, 27*), pretreatment with monensin also blocked EGF-induced EGFR degradation, whereas chloroquine had negligible effects on AR-induced EGFR recycling (Figure S1 of the Supporting Information). To provide numerical support for our colocalization observations, the colocalization coefficients, which measure pixel overlap between the EGFR and EEA1, Rab11, and LAMP in Figure 3A,B,D, were quantified using Zeiss LSM 510 colocalization analysis software (Figure 3E). Taken together, the results presented in Figure 3 provide unequivocal evidence that under synchronized conditions, AR triggers normal EGFR endocytosis, as previously observed for EGF (*3, 13, 22*−*25*). More importantly, we demonstrate that although both ligands induce similar levels of early EGFR autophosphorylation, EGF and AR cause differential trafficking of EGFRs.

Effects of AR and EGF on EGFR Ubiquitination and Association with c-Cbl. It was previously shown that c-Cbl-mediated ubiquitination plays a central role in the lysosomal sorting and degradation of the EGFR (*3, 9*−*15*). Thus, we hypothesized that the observed differences in the intracellular trafficking of the EGFR upon stimulation with EGF or AR might result from differential association of the EGFR with c-Cbl and/or differential receptor ubiquitination by c-Cbl, as previously observed for TGF-α (*3*). To study these possibilities, we first examined the activation of c-Cbl after various periods of EGF and AR treatment in HEK293 cells. As demonstrated in Figure 4A, although both ligands induced considerable tyrosine phosphorylation of c-Cbl (a surrogate of its activity), EGF evoked more robust and sustained responses than AR. Figure 4B shows results from co-immunoprecipitation experiments in which c-Cbl was immunoprecipitated from HEK293 cells, following which Western blotting of the EGFR was performed. Those results show similar amounts of the EGFR in c-Cbl immunoprecipitates after a 2 min treatment with either ligand. However,

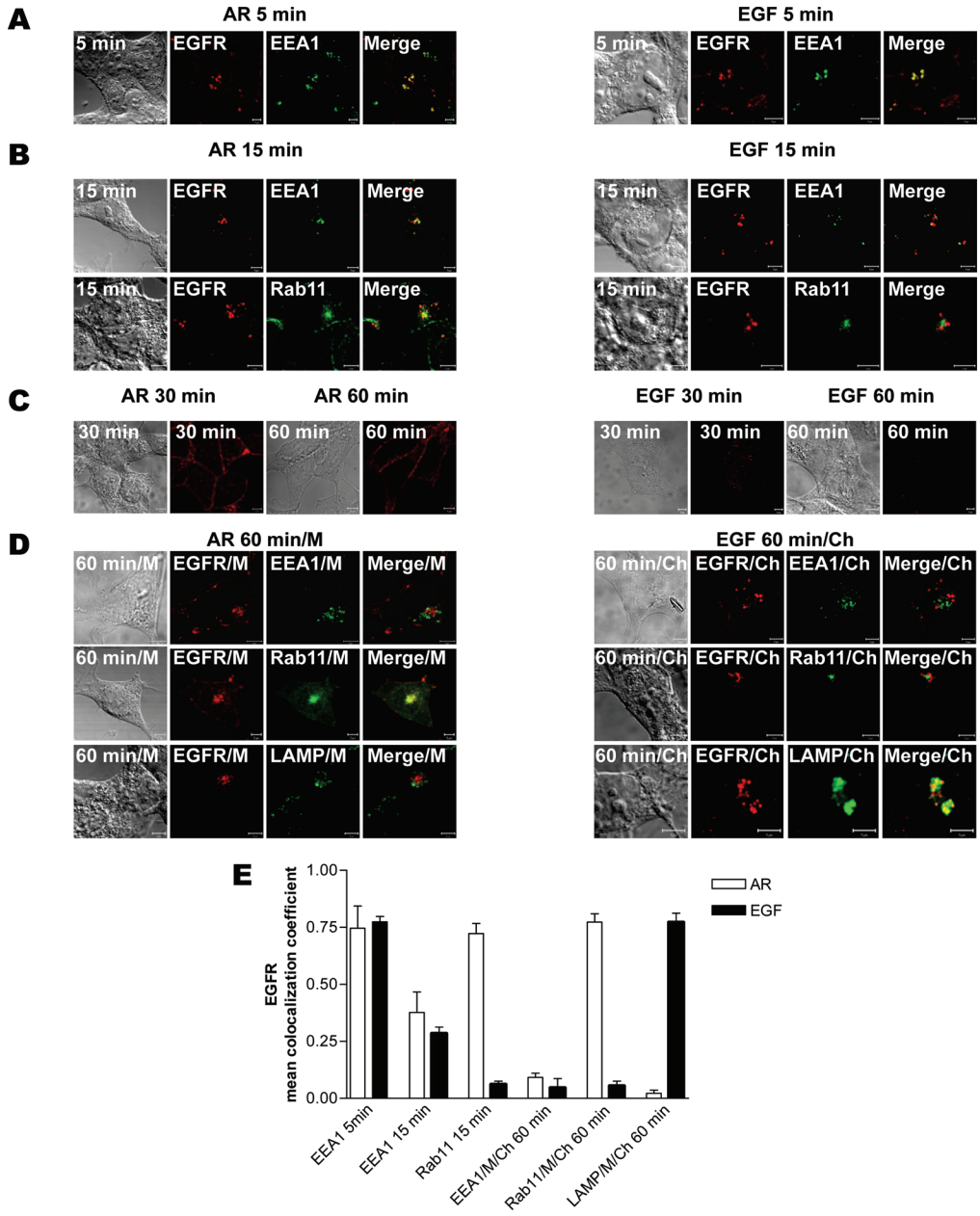

FIGURE 3: Differential trafficking of the EGFR induced by EGF and AR. Serum-deprived HEK293 cells were pretreated without (panels A−C) or with 100 μM chloroquine (Ch) or 100 μM monensin (M) for 15 min (panel D), incubated on ice with 1 ng/mL EGF or 100 ng/mL AR for 45 min, washed free of unbound ligand, warmed, and exposed to prewarmed ligand-free medium at 37 °C for 5 (A), 15 (B), or 30 and 60 min (C and D). The cells then were fixed, stained with anti-EGFR antibody [visualized with Alexa Fluor 568-conjugated secondary antibody (red)] and anti-EEA1, -Rab11, or -LAMP antibodies [visualized with Alexa Fluor 488-conjugated secondary antibody (green)], and analyzed by confocal microscopy. Data shown are representative of three independent experiments. Yellow indicates colocalization. The bar is 5 μm. (E) The colocalizations between the EGFR and EEA1, Rab11, or LAMP observed in panels A, B, and D were quantified using Zeiss LSM 510 META colocalization analysis software. The mean colocalization coefficients, averaged from at least three independent single-cell images, represent pixel overlap between the EGFR and EEA1, Rab11, or LAMP. The coefficients vary from 0 to 1, with 0 corresponding to nonoverlapping images and 1 corresponding to 100% colocalization.

the association between c-Cbl and EGFR was transient in cells treated with AR in that there was virtually no co-immunoprecipitation after 5 and 15 min. In marked contrast, the association between c-Cbl and the EGFR was more stable in cells treated with EGF, persisting without diminishing for 15 min. The pattern of EGFR co-immunoprecipitation with c-Cbl in Figure 4B was closely recapitulated when we probed ubiquitination of the EGFR at various time points after stimulation with EGR or AR (Figure 4C). The fact that AR-induced c-Cbl phosphorylation lasted longer (Figure 4A) than AR-induced association between c-Cbl and the EGFR or EGFR ubiquitination (panels B and C of Figure 4, respectively) suggests that deubiquitinating enzymes acted on the EGFR prior to dephosphorylation and inactivation of c-Cbl.

Role of c-Cbl in AR-Induced EGFR Trafficking. To examine the potential importance of c-Cbl in recycling of the EGFR, we used an siRNA knockdown approach, in which a pool of four different siRNA duplexes was utilized to selectively deplete endogenous c-Cbl. We analyzed the effectiveness of siRNA against c-Cbl by means of Western blotting analysis and confirmed that c-Cbl protein expression could be suppressed considerably (Figure 5A). We then assessed the ability of AR to induce EGFR ubiquitination under control and c-Cbl-knockdown conditions (Figure 5B). Con-

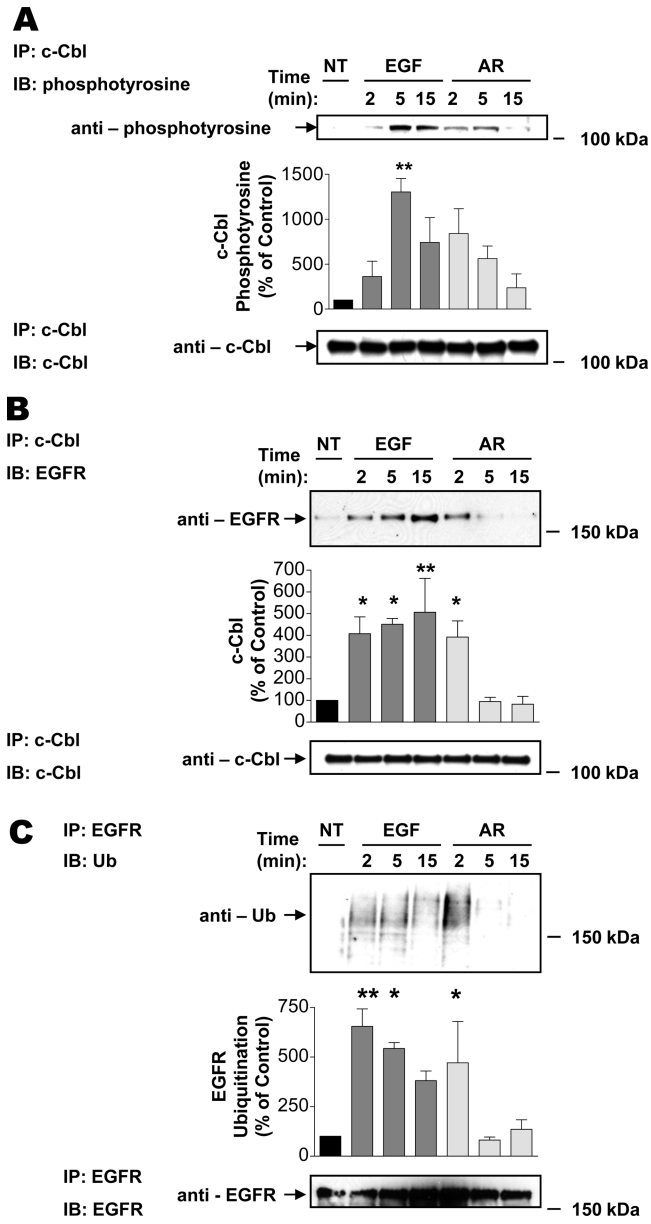

FIGURE 4: Effects of AR and EGF on EGFR ubiquitination and association with c-Cbl. Serum-deprived HEK293 cells were treated at 37 °C with vehicle (NT), 1 ng/mL EGF, or 100 ng/mL AR for 2, 5, or 15 min. After being washed with ice-cold PBS, cell lysates were (A and B) subjected to immunoprecipitation with an antibody to c-Cbl, followed by immunoblotting with an antibody to phosphotyrosine or the EGFR, or (C) subjected to immunoprecipitation with an antibody to the EGFR, followed by immunoblotting with an antibody to ubiquitin. Blots were then stripped and reprobed for the total amount of c-Cbl or the EGFR. Insets are representative of three independent experiments. Results are means ± SE (n = 3). One asterisk indicates a p of <0.05 vs vehicle (NT). Two asterisks indicate a p of <0.01 vs vehicle (NT).

sistent with previous reports (*13*), Cbl deficiency significantly reduced the level of EGFR ubiquitination. To test the possibility that c-Cbl is involved in AR-induced EGFR trafficking, we exposed control and c-Cbl-knockdown cells to AR and determined the extent of receptor recycling by means of radioligand binding (Figure 5C). These experiments demonstrate that similar levels of EGFR were endocytosed 15 min after a synchronous pulse with AR in control and c-Cbl-knockdown cells. Interestingly, however, receptor recycling in c-Cbl-depleted cells was significantly delayed compared with control cells (Figure 5C, c-Cbl siRNA, 30 min). Importantly, it should be noted that similar observations were made in HEK293 cells, in which both c-Cbl and Cbl-b were simultaneously depleted (Figure S2 of the Supporting Information). To confirm the requirement for c-Cbl ubiquitin ligase activity in AR-induced receptor recycling, we used a dominant negative mutant of c-Cbl, named Cbl-N, which lacks regions required for c-Cbl-mediated EGFR ubiquitination (*17*). Consistent with our c-Cbl knockdown results, we demonstrated that overexpression of the Cbl-N mutant inhibited ubiquitination of the AR-stimulated EGFR (Figure 5E) and delayed AR-induced EGFR recycling (Figure 5F). These results suggest that the ubiquitin ligase activity of c-Cbl is important for recycling of the AR-stimulated EGFR.

To characterize in greater detail the trafficking of AR-stimulated EGFR in c-Cbl-knockdown cells, we performed side-by-side comparisons of trafficking in HEK293 cells with little silencing of c-Cbl (cells without an asterisk) and those with marked silencing (cells with an asterisk) (Figure 6). Having established that under synchronized conditions the AR-activated EGFR undergoes endocytosis (Figures 3 and 5C), we analyzed the localizations of AR-induced EGFR in control and c-Cbl-knockdown cells (Figure 6B−D). As shown in Figure 6B, following a 15 min chase with AR, internalization of the EGFR was apparent (evidenced by localization of the EGFR in intracellular vesicles) regardless of whether c-Cbl expression was knocked down. To further investigate the roles for c-Cbl in postendocytotic, AR-induced EGFR trafficking, we examined the localization of the EGFR 30 min after a synchronized ligand pulse in c-Cbl-depleted cells (Figure 6C,D). We observed that c-Cbl knockdown greatly diminished the level of AR-induced EGFR recycling, as evidenced by intracellular accumulations of the EGFR in cells in which c-Cbl was knocked down (Figure 6C, cell with an asterisk), as compared with those in which c-Cbl was not knocked down, in which there was localization of the EGFR at the cell surface (Figure 6C, cell without an asterisk). It should be noted that similar findings were observed when the control and c-Cbl-knockdown cells were continuously treated with AR for 30 min (data not shown). Figure 6D shows representative photomicrographs of the intracellular accumulation of the EGFR in the early endosomes of cells treated with c-Cbl siRNA 30 min following a synchronized pulse of AR, as evidenced by clear colocalization of the EGFR with the early endosomal marker EEA1, and no overlap with Rab11. This should be contrasted with Figure 3C, in which EGFRs were localized to the plasma membrane 30 min after a pulse of AR. These results strongly suggest that c-Cbl is required for efficient exit of the recycling EGFR from endosomes.

Role of c-Cbl in AR-Induced Signaling. Because one of the main EGFR-mediated signals is activation of the ERK family of MAPKs (*28*), we focused our attention on potential roles of c-Cbl in ligand-induced ERK1/2 MAPK phosphorylation. Figure 7 compares the ability of AR to induce ERK1/2 MAPK phosphorylation in control and c-Cbl-knockdown cells. Under both conditions, phosphorylation of ERK1/2 MAPK peaked at ~500% by 5 min, but the signal was more sustained in c-Cbl-knockdown cells. When similar experiments were performed using EGF, ERK phosphorylation was much more pronounced than for AR (approximately 1500%) in cells treated with scrambled siRNA.

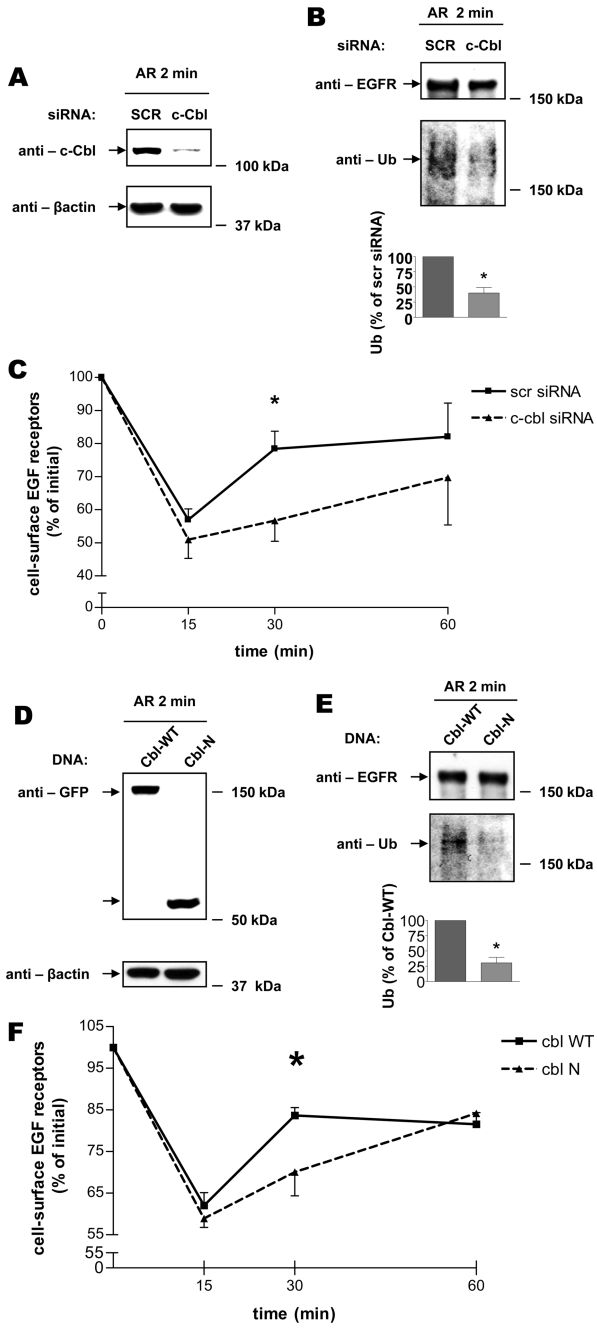

FIGURE 5: Role of c-Cbl in AR-induced EGFR recycling. Serum-deprived HEK293 cells, which had been transiently transfected with scrambled (SCR) or c-Cbl siRNA for 72 h (A−C) or which had been transiently transfected with GFP-c-Cbl-WT or GFP-c-Cbl-N for 24 h (D−F), were (A and D) treated with 100 ng/mL AR for 2 min and extracted with RIPA buffer, following which cell lysates were immunoblotted with anti-c-Cbl, -GFP, or -β-actin antibodies; (B and E) preincubated with sulfo-NHS-biotin for 30 min to label cell surface proteins and subsequently treated with 100 ng/mL AR for 2 min (after being washed with ice-cold PBS, biotinylated proteins were analyzed by SDS−PAGE followed by immunoblotting with antibodies to EGFR and Ub); or (C and F) incubated on ice with 100 ng/mL AR for 45 min and subsequently incubated in prewarmed ligand-free medium at 37 °C for 0, 15, 30, or 60 min. The cells were then rinsed with ice-cold binding buffer, followed by a 7 min incubation with a low-pH stripping buffer. Specific binding was assessed by incubating cells for 90 min on ice with [^{125}I]EGF. The results are expressed as a percentage of the original binding sites measured at 0 min. Data shown in panels A, B, D, and E are representative of three independent experiments. Results in panels C and F are means ± SE of three separate experiments. An asterisk indicates a p of <0.05 vs scrambled siRNA.

ERK phosphorylation was similar in magnitude in c-Cbl-knockdown cells, and phosphorylation was slightly prolonged (Figure S3A of the Supporting Information). It should be noted that, as predicted, the EGF-induced degradation of EGFR in our system was significantly blocked in c-Cbl-depleted cells (Figure S3B of the Supporting Information). Taken together, these results strongly suggest that c-Cbl attenuates later phases of EGFR-induced ERK1/2 MAPK activation.

DISCUSSION

The EGFR has been shown to play key roles in the regulation of many normal cellular processes. Despite significant advances, our knowledge about the cellular trafficking and signaling induced by various cognate EGFR ligands is incomplete. In this study, we investigated the roles of the ubiquitin ligase c-Cbl in EGFR trafficking and signaling after stimulation with AR, a cognate EGFR ligand that induces receptor recycling. What is new about this work is that our findings implicate c-Cbl as a critical mediator of active sorting of the EGFR to the recycling endosomes. To the best of our knowledge, this is the first demonstration that c-Cbl mediates the efficient exit of the EGFR from early endosomes into recycling pathways. Our results also illustrate a potential role for c-Cbl in controlling the temporal dynamics (transient vs sustained) of ERK1/2 MAPK phosphorylation by the EGFR.

The family of EGFR ligands has expanded throughout the years and now includes seven members, i.e., EGF, HB-EGF, BTC, TGF-α, AR, EPR, and EPG. These ligands variably induce degradation or recycling of the EGFR. Although the mechanisms underlying EGF-induced downregulation of the EGFR continue to be carefully studied, much less is known regarding the trafficking of EGFR following stimulation with other members of this family. A recent study by Stern et al. (*4*) investigated the effects of AR and BTC on the EGFR. The results suggested that AR did not lead to EGFR degradation, but the effects on recycling were not examined. Those studies were performed on cells overexpressing EGF receptors exposed to supraphysiological concentrations of the ligands. Although experiments with ectopic expression of EGFR present several technical advantages, their interpretation is problematic due to the fact that endocytosis and lysosomal sorting are saturable processes (*1, 29, 30*) and that receptor overexpression can disrupt normal trafficking (unpublished observation). Therefore, we investigated the effects of AR and EGF on the endogenous EGFR expressed in HEK293 cells. We demonstrate herein differential intracellular trafficking of the EGFR following stimulation with those ligands. Following endocytosis, the receptors enter early endosomes where they undergo sorting to either recycling or degradative fates (*31*). In that regard, our results suggest that when EGF and AR are applied in concentrations that result in equivalent levels of short-term EGFR phosphorylation, EGF targets the EGFR to degradation, whereas AR targets the EGFR to a recycling pathway.

With regard to recycling, it is known that the internalized EGFR can recycle back to the plasma membrane directly from early endosomes through Rab4-positive endosomes, or from perinuclear Rab11-positive recycling endosomes (*32, 33*). Although it was not our intention to directly address this

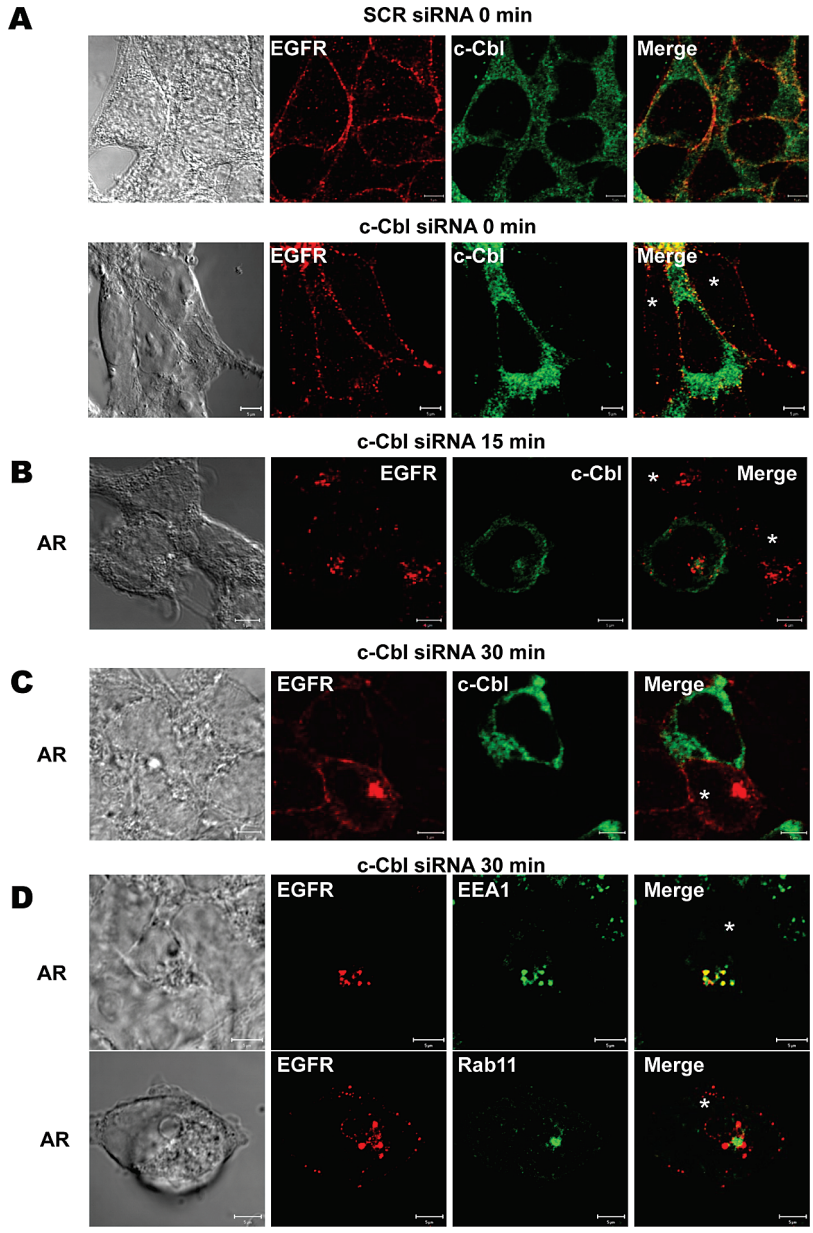

FIGURE 6: Role of c-Cbl in AR-induced EGFR trafficking. Serum-deprived HEK293 cells, which had been transiently transfected with scrambled (SCR) or c-Cbl siRNA for 72 h, were incubated on ice with 100 ng/mL AR for 45 min, washed free of unbound ligand, and subsequently incubated in prewarmed ligand-free medium at 37 °C for 0 (A), 15 (B), or 30 min (C). The cells then were fixed, stained with anti-EGFR [visualized with Alexa Fluor 568-conjugated secondary antibody (red)] and anti-c-Cbl [visualized with Alexa Fluor 488-conjugated secondary antibody (green)] antibodies, and analyzed by confocal microscopy. Fields were chosen to show simultaneously cells in which c-Cbl was depleted and cells in which c-Cbl was not, to facilitate direct comparisons. White asterisks show positions of c-Cbl-depleted cells. (D) These micrographs show the intracellular localization of the EGFR [visualized with Alexa Fluor 568-conjugated secondary antibody (red)], in serum-deprived HEK293 cells, which had been transiently transfected with scrambled (SCR) or c-Cbl siRNA for 72 h, 30 min after a synchronized pulse with AR. Dual labeling was performed with markers of subcellular organelles, i.e., early endosomes and the perinuclear recycling compartment [visualized with anti-EEA1 and -Rab11 antibodies, respectively, and Alexa Fluor 488-conjugated secondary antibody (green)]. White asterisks show the positions of c-Cbl-depleted cells. Yellow indicates colocalization. The bar is 5 μm. Data shown are representative of at least three independent experiments.

matter, our results indicated that the AR-activated EGFR returned to the plasma membrane predominantly from the perinuclear area. It should be noted, however, that according to emerging models, endosomes can be viewed as a mosaic of distinct but interconnected domains containing different combinations of the Rab family of small GTPases (reviewed in ref *34*).

There are at least three possible mechanisms that could explain why AR stimulation results in recycling, rather than EGFR degradation and downregulation. It is possible that AR could induce heterodimerization with other members of the ErbB family of receptors, in a fashion distinct from that of EGF (*35*). In that regard, HEK293 cells express endogenous levels of all four ErbB receptors (data not shown). Another possibility is that the AR−EGFR ligand−receptor interaction is weaker than the EGF−EGFR interaction within the acidic environment of endosomes (*2*). A third possibility is that AR is ineffective for inducing c-Cbl-mediated EGFR ubiquitination (*12*). Because Johnson et al. (*36*) provided strong evidence that AR functionally coupled primarily to the EGFR, we felt it was unlikely that AR triggered EGFR−ErbB2 or −ErbB3 heterodimerization. Although AR

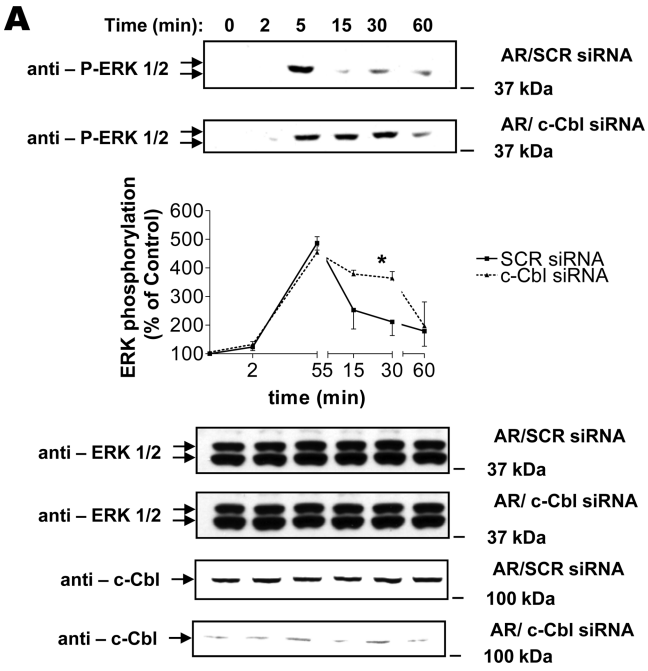

FIGURE 7: Role of c-Cbl in AR-induced MAPK activation. Serum-deprived HEK293 cells, which had been transiently transfected with scrambled (SCR) or c-Cbl siRNA for 72 h, were incubated at 37 °C with 100 ng/mL AR for 0, 2, 5, 15, 30, and 60 min. After being washed with ice-cold PBS, cells were extracted with RIPA buffer, following which cell lysates were immunoblotted with anti-phospho-ERK1/2. Blots then were stripped and reprobed for the total amount of ERK1/2 or c-Cbl. Results are means ± SE ($n = 3$). An asterisk indicates a p of <0.05 vs scrambled siRNA. Insets are representative of three independent experiments.

has been shown to have a much lower affinity for the EGFR than EGF does (*20*), pH-sensitive binding of AR to the EGFR has not yet been investigated. Inasmuch as we normalized the concentrations of AR and EGF to similar levels of phosphorylation of the EGFR, we did not further investigate the stability of AR−EGFR and EGF−EGFR binding in intracellular vesicles. Instead, we focused our attention on the possibility that AR and EGF induce a differential engagement with c-Cbl. Indeed, AR clearly induced only a transient association of the EGFR with c-Cbl and subsequently less sustained receptor ubiquitination, supporting a possible role for the sustained EGFR ubiquitination in the efficient receptor sorting to lysosomal degradation. It should be noted that our results are consistent with those with ectopic expression of the EGFR and supraphysiological concentrations of AR (up to 136 nM), in which decreased levels of receptor degradation, ubiquitination, and association with c-Cbl also were observed (*4*). Together, our data and those of others (*3, 4*) suggest that AR and TGF-α use common mechanisms to modulate receptor recycling.

Although our data that show that AR (like TGF-α) induces transient association of the EGFR with c-Cbl and receptor ubiquitination point to potential roles of c-Cbl in the trafficking and signaling of recycling EGF receptors, these possibilities heretofore have received little attention. Despite considerable debate in the literature regarding the role of c-Cbl in endocytosis (*9, 13*−*15, 23, 37*−*41*), one still might speculate that c-Cbl is required for AR-induced EGFR internalization. Recent work by Huang et al. (*15*), demonstrating a mutational analysis of EGFR lysine residues, suggests that a Cbl-mediated ubiquitination event is involved in EGFR internalization, yet the ubiquitination of the receptor itself is not necessary. Our results document that substantial knockdown of c-Cbl does not interfere with AR-induced EGFR internalization in native HEK293 cells (Figure 5C). In light of recent findings suggesting that both c-Cbl and Cbl-b must be depleted simultaneously for detection of the effect of siRNAs on EGFR endocytosis (*9*), we also investigated whether double knockdown of c-Cbl and Cbl-b could interfere with AR-induced EGFR endocytosis (Figure S2 of the Supporting Information). Neither condition appeared to significantly affect receptor endocytosis. It should be noted, however, that our results do not rule out the possibility that a residual pool of c-Cbl/Cbl-b is sufficient to support EGFR endocytosis or that Cbl-c could facilitate EGFR endocytosis (*38*).

In any case, the most critical observation made was that knockdown of c-Cbl (Figure 5C and Figure S2C of the Supporting Information), as well as overexpression of a c-Cbl dominant negative mutant (Figure 5F), altered receptor recycling to the plasma membrane. Our findings challenge the current dogma that the role of Cbl in EGFR sorting is limited merely to the degradative pathway. We demonstrated herein that c-Cbl knockdown and an inactive c-Cbl mutant delay sorting of the AR-activated EGFR to the recycling endosomes. Because the AR-activated EGFR was delayed in the early endosomes in c-Cbl-depleted cells, it appears that c-Cbl is required for EGFR to efficiently exit from these endosomal vesicles. Taken together, we speculate that c-Cbl has the ability to govern the AR-induced active sorting of EGFR from the early endosomes to recycling endosomes.

The results presented here suggest at least two mechanisms by which c-Cbl can regulate active sorting of receptors to recycling endosomes. It is possible that the AR-induced transient EGFR ubiquitination is required at the initial checkpoint to "presort" constitutively trafficked unliganded EGFR from liganded receptors that are further sorted to degradative or recycling pathways. It should be noted, however, that although our studies strongly point to a critical role for the ubiquitin ligase activity of c-Cbl in EGFR recycling, our studies do not warrant that initial AR-induced EGFR ubiquitination is involved in the active sorting of the EGFR to recycling endosomes. A systematic mutational analysis of EGFR lysine residues (*15*) is essential for addressing this issue. On the other hand, our results demonstrating that AR-induced c-Cbl phosphorylation (a surrogate of its activity) lasted longer than ubiquitination of the EGFR (panels A and B of Figure 4, respectively) suggest an alternative or more complex mechanism.

Alternatively, perhaps, c-Cbl interacts with or regulates key vesicular components that are integral for active recycling. In that regard, Hanyaloglu et al. (*42*) identified a novel function of hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) in mediating recycling of certain G protein-coupled receptors. They reported that this function was distinct from a previously defined role of Hrs in lysosomal sorting (*43*), as it did not require receptor ubiquitination. They also hypothesized that Hrs may mediate distinct sorting functions depending upon whether receptors were ubiquitinated or non-ubiquitinated. Additionally, Stern et al. (*44*) have shown that c-Cbl modulates Hrs ubiquitination, phosphorylation, and protein levels, thus controlling the composition of the sorting machinery. It is tempting to

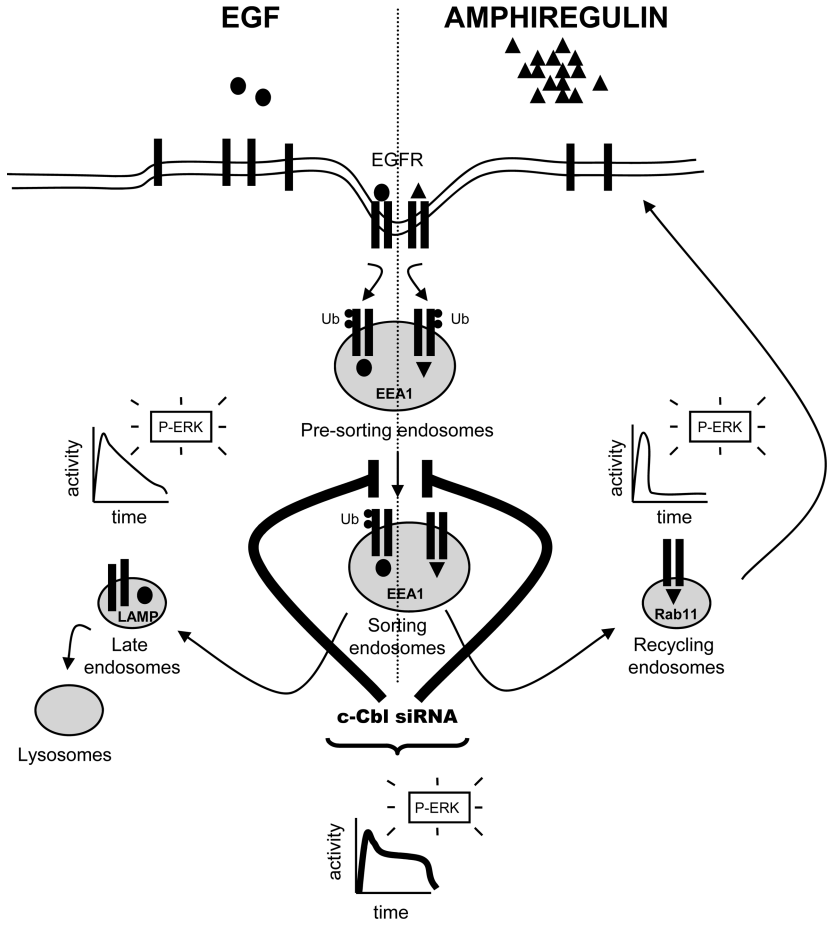

FIGURE 8: Roles of c-Cbl in EGFR recycling and signaling. Binding of AR (▲; right side of the scheme) or EGF (●; left side of the scheme) to the EGFR triggers receptor endocytosis. Following internalization into early presorting endosomes (EEA1-positive), the AR- or EGF-stimulated EGFR is sorted into recycling endosomes (Rab11-positive) or late endosomes and lysosomes (LAMP-positive), respectively. Differential trafficking of the EGFR correlates with differential patterns of ERK activation; i.e., unlike AR, which causes transient phosphorylation of ERK, EGF results in much more persistent activation of ERK. AR induces transient ubiquitination of the EGFR (•, Ub), whereas EGF induces more sustained ubiquitination. Regardless of EGFR ligand, c-Cbl regulates exit into both recycling and degradative EGFR trafficking pathways. In the absence of c-Cbl, the disrupted sorting of EGFR causes receptor retention within the early endosomes, which consequently is associated with more sustained phosphorylation of ERK.

speculate that c-Cbl may conspire with Hrs to determine whether EGFRs exit early endosomes into recycling and/or degradative compartments. Taken together, our investigation raises an intriguing possibility that c-Cbl regulates sorting of the EGFR to recycling pathways. Future studies are necessary to better understand the molecular mechanisms underlying this process.

Although c-Cbl has been implicated in the activation of various MAPKs in response to cell surface receptor activation (*16*), limited information is available regarding the role of c-Cbl in EGFR-mediated MAPK signaling. Our results presented here (Figure S3A of the Supporting Information) are consistent with those from other groups, which have reported that compromising ubiquitin ligase activity of c-Cbl leads to enhanced EGF-stimulated ERK1/2 MAPK activation (*45*−*47*). However, the potential role of c-Cbl in the activation of ERK1/2 MAPK induced by ligands that do not cause EGFR degradation has not yet been explored. In this report, we show that AR induced much more sustained ERK1/2 MAPK phosphorylation in c-Cbl-knockdown cells, as compared with control cells. This finding is significant in that recent studies have indicated that small changes in the strength and/or duration of ERK1/2 MAPK signaling may evoke strikingly different responses, e.g., cytoplasmic versus nuclear localization of ERK1/2 MAPK, differential gene expression, or proliferation versus differentiation (reviewed in refs *48* and *49*). Given the fact that aberrant endosomal trafficking and EGFR signaling have been linked to a number of diseases, including cancer (*50*), the roles of c-Cbl in trafficking and signaling of the recycling EGF receptors are worthy of closer examination.

In summary, in this study, we addressed several fundamental issues regarding the processing, sorting, and signaling of the EGFR following stimulation with AR (Figure 8). Our study points to a potential global role for c-Cbl in regulating vesicular sorting of the EGFR, regardless of whether the receptors have been destined for recycling or degradation. It appears that the function of c-Cbl is not merely limited to the regulation of receptor degradative pathways. Our work supports additional roles for c-Cbl in (1) mediating differential effects of EGF and AR on the EGFR, (2) controlling efficient exit of the EGFR from EEA1-positive endosomes into a recycling pathway, and (3) controlling the kinetics of EGFR-stimulated ERK1/2 MAPK. In the future, it will be of great interest to investigate the mechanisms via which c-Cbl regulates trafficking and signaling of the recycling EGFR.

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SUPPORTING INFORMATION AVAILABLE

Differential trafficking of EGFR induced by AR and EGF (Figure S1), roles of c-Cbl and Cbl-b in AR-induced EGFR recycling (Figure S2), and role of c-Cbl in EGF-induced EGFR degradation and MAPK activation (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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