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Critical Role of ESCRT Machinery in EGFR Recycling[†]

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ABSTRACT: The molecular mechanisms of EGFR vesicular trafficking to lysosomes have recently received considerable attention. It is now clear that endosomal sorting complexes required for transport (ESCRTs) are critical for EGFR degradation. Although an increasing number of membrane receptors also undergo recycling via specific pathways, little information is available regarding regulated recycling of EGFR. In this study, we investigated the roles of ESCRTs in EGFR recycling after stimulation with amphiregulin (AR). We used ESCRT small interfering RNA (siRNA) duplexes to demonstrate that AR-induced EGFR intracellular processing involves active sorting to the recycling pathway through specific members of the ESCRT family.

The epidermal growth factor (EGF)[1] receptors (EGFRs) belong to the ErbB family of cell surface receptor tyrosine kinases (RTKs). EGFRs are among the most extensively studied receptors because of their essential roles in both normal cellular functions and the pathogenesis of human cancers. Many different growth factors serve as ligands for the EGFR; these include EGF, transforming growth factor-α, heparin-binding EGF, betacellulin, amphiregulin (AR), epiregulin, and epigen. Ligand binding to the EGFR induces autophosphorylation through activation of EGFR tyrosine kinase activity. Following activation, the EGFR undergoes internalization and endocytic trafficking. Some receptors recycle back to the plasma membrane, whereas others commit to a degradative pathway via late endosomes and lysosomes. In that regard, EGF-induced EGFR trafficking to lysosomes serves as a prototype for RTK sorting to the degradative pathway (*1*). It has become evident that c-Cbl ubiquitin ligase-mediated EGFR ubiquitination is a prerequisite of selective trafficking of EGFRs to lysosomes. ESCRT-0, -I, -II, and -III complexes, which recognize and capture the ubiquitinylated cargo in the membranes of sorting endosomes, also have emerged as crucial components of EGFR lysosomal targeting and degradation (*2*). Despite significant advances, however, whether ESCRT complexes function sequentially according to their numerical order (*2*), form concentric rings around ESCRT-0 (*3*), or perhaps function independently at different stages of endosomal sorting remains unknown. In contrast to ubiquitin-tagged receptors destined for degradation, unliganded and nonubiquitinated liganded receptors are recycled back to the plasma membrane through two distinct endosomal compartments, i.e., the sorting or recycling endosomes (*4*). Interestingly, evidence suggesting that recycling of some G protein-coupled receptors is specifically regulated is emerging (*5*). With regard to EGFR trafficking, however, it is thought that in the absence of targeting information, e.g., specific protein sequences, post-translational modifications, and/or structural features, receptors are transported back to the plasma membrane through bulk flow (*4*). The functions of ESCRTs in sorting nonubiquitinated and/or recycling EGF receptors are yet to be elucidated.

Previously, we documented a global role for c-Cbl in regulating vesicular sorting of EGFR (*6*). In this work, we turned our attention to the potential involvement of ESCRT complexes in recycling of the EGFR. We used a siRNA knockdown approach to selectively deplete c-Cbl and representative components of ESCRT-0, -I, -II, and -III, i.e., Hrs, Tsg101, Vps25, and Vps24, respectively. We analyzed the effectiveness of siRNA against c-Cbl and ESCRT proteins by means of Western blotting analysis and confirmed that expression of c-Cbl, Hrs, Tsg101, Vps25, and Vps24 could be selectively suppressed (Figure 1A and Figure S1 of the Supporting Information). We then assessed the ability of EGF to induce EGFR degradation under control, c-Cbl-knockdown, and ESCRT-knockdown conditions. Although the requirement for ESCRT-II in EGFR degradation is still a subject of debate (*1*), our results demonstrate that all ESCRTs, similar to c-Cbl, are required for efficient EGFR degradation (Figure 1B); these are consistent with those reported by Raiborg et al. (*7*). AR had negligible effects on EGFR degradation (Figure 1C). To test the possibility that ESCRTs play a role in AR-induced EGFR recycling, we exposed control, c-Cbl-knockdown, and ESCRT-knockdown cells to AR and assessed the extent of EGFR recycling using radioligand binding (Figure 2). This revealed that similar levels of EGFR were internalized 15 min after a synchronous pulse with AR in control, c-Cbl-knockdown, and ESCRT-knockdown cells. In striking contrast, EGFR recycling in ESCRT-I- and -III-depleted cells was significantly delayed compared with that in control cells [Figure 2A, Tsg101 (ESCRT-I)- and Vps24 (ESCRT-III)-depleted cells, 30 min]. Surprisingly, although ESCRT-0 and -II were essential for EGFR degradation (Figure 1B and ref *7*), these complexes appeared to be dispensable for EGFR recycling [Figure 2B, Hrs (ESCRT-0) and Vps-25 (ESCRT-II), 30−60 min]. Importantly, the effects of

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[1]Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase; AR, amphiregulin; ESCRT, endosomal sorting complex required for transport; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

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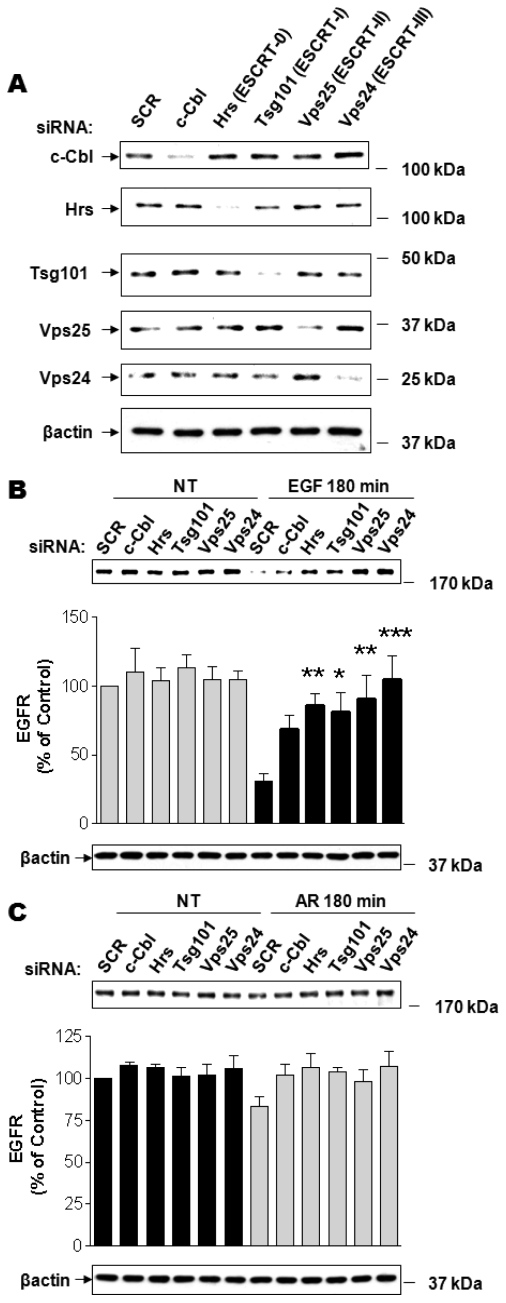


FIGURE 1: Role of ESCRTs in ligand-induced EGFR degradation. Serum-deprived HEK293 cells, which had been transiently transfected with control scrambled (SCR), c-Cbl, Hrs (ESCRT-0), Tsg101 (ESCRT-I), Vps25 (ESCRT-II), or Vps24 (ESCRT-III) siRNA for 72 h, were (A) untreated, (B) treated with vehicle (NT) or 1 ng/mL EGF, or (C) treated with vehicle (NT) or 100 ng/mL AR for 180 min. Cells were extracted with RIPA buffer, after which cell lysates were immunoblotted with (A) anti-c-Cbl, -Hrs, -Tsg101, -Vps25, -Vps24, or -β-actin antibodies and (B and C) anti-EGFR or -β-actin antibodies. Results are means ± the standard error ($n = 4$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs control scrambled (SCR) siRNA. Blots shown are representative of four independent experiments.

ESCRT-I and -III appear to be specific for AR-induced EGFR recycling in that knockdown of ESCRTs did not affect trafficking of the constitutively recycling transferrin receptor (Figure S2 of the Supporting Information). It should be noted that our results also document that substantial knockdown of c-Cbl did not interfere with AR-induced EGFR internalization but significantly delayed EGFR recycling in native HEK293 cells (Figure 2A, c-Cbl siRNA), as previously observed and thoroughly discussed by us in ref *6*.

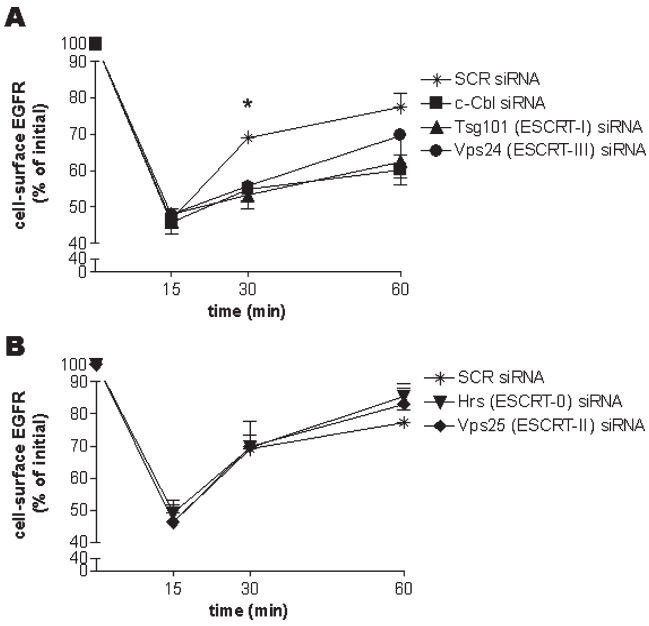
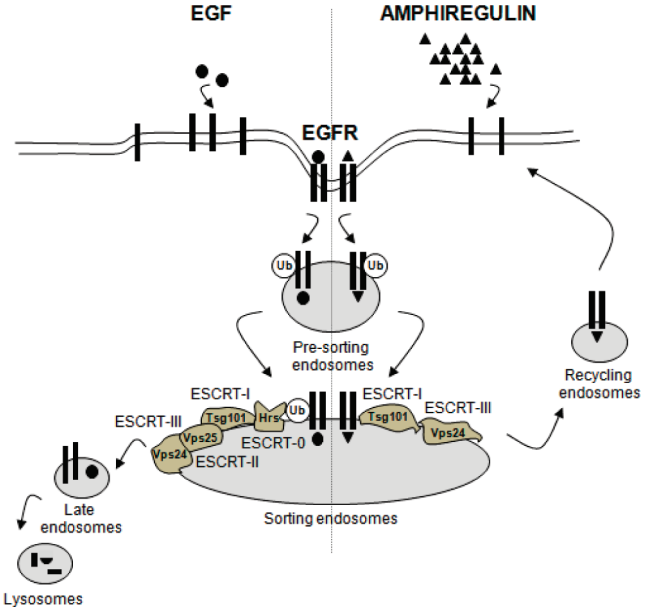


FIGURE 2: Role of ESCRTs in AR-induced EGFR recycling. Serum-deprived HEK293 cells, which had been transiently transfected with (A) control scrambled (SCR), c-Cbl, Tsg101 (ESCRT-I), or Vps24 (ESCRT-III) siRNA or (B) control scrambled (SCR), Hrs (ESCRT-0), or Vps25 (ESCRT-II) siRNA for 72 h, were incubated on ice with 100 ng/mL AR for 45 min and subsequently incubated in pre-warmed, ligand-free medium at 37 °C for 0, 15, 30, or 60 min. Cells then were rinsed 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. Results are means ± the standard error ($n = 4$); *$p < 0.05$ vs control scrambled (SCR) siRNA. Data shown were averaged from four independent experiments.

Our previous findings demonstrated that c-Cbl ubiquitin ligase is required for sorting of AR-activated EGFR to the recycling pathway and challenged the prevailing dogma that the role of Cbl in EGFR sorting was limited merely to the degradative pathway (*6*). Because AR induced only transient EGFR ubiquitination, we proposed that the AR-induced EGFR ubiquitination was critical to "presort" unoccupied, nonubiquitinated EGFR from occupied receptors that were subsequently sorted to recycling or degradative pathways. Our data presented herein demonstrate that ESCRT-I and -III, but not ESCRT-0 or -II, are essential for AR-induced EGFR recycling. In that regard, it is noteworthy that ESCRT-II also has been reported to be dispensable for other nonendosomal ESCRT functions. For instance, a unique assembly of ESCRT-I and -III has been reported to play a role in mammalian cytokinesis as well as budding of viruses such as HIV-1 (*2*). It is possible then that altered ESCRT composition and/or levels also could give rise to fundamentally different endosomal functions of ESCRTs. Nevertheless, on the basis of the kinetics of AR-induced EGFR ubiquitination (~2 min) (*6*), we speculate that receptor ubiquitination is not essential for the ESCRT-mediated sorting to the recycling pathway. However, a systematic mutational analysis of EGFR lysine residues (*8*) is essential for addressing this issue. In light of our current findings demonstrating that ESCRT-0 and -II are dispensable for efficient AR-induced EGFR recycling, it is tempting to speculate that, depending upon whether EGFRs are destined for degradation or recycling back to the plasma membrane, ESCRT complexes could serve differential functions.

Scheme 1: Differential Role of ESCRTs in EGFR Trafficking[a]



[a]Binding of EGF (●; left side of the scheme) or AR (▲; right side of the scheme) to the EGFR triggers receptor endocytosis. AR induces transient EGFR ubiquitination (Ub), whereas EGF induces more sustained ubiquitination. Regardless of EGFR ligand, c-Cbl regulates the exit of the EGFR into sorting endosomes, where EGFR undergoes sorting to either recycling or degradative fates. Whereas all ESCRTs are essential for EGFR degradation, ESCRT-0 and -II are dispensable for efficient EGFR recycling.

Although evidence that receptor trafficking and signaling are functionally interrelated is accumulating (*9*), this relationship still remains obscure. Recent studies indicate that EGFRs remain active within the endosomes and that endocytic mechanisms control EGFR signaling (*6*, *10*). In that regard, it is possible that the kinetics of recycling of EGFR controls the magnitude or duration of its signaling. In that regard, it appears that small changes in the strength and/or duration of extracellular signal-regulated kinase 1/2 (ERK1/2) MAPK signaling could evoke strikingly differential cellular responses, i.e., proliferation versus differentiation (*11*). Additionally, it now is evident that aberrant EGFR trafficking is associated with uncontrolled signaling and frequently with cancer.

What is new about this work is that we have implicated members of the ESCRT-I and -III complexes in regulating the rate of recycling of AR-stimulated EGFR. In this study, we addressed several fundamental aspects of EGFR sorting following stimulation with AR (Scheme 1). Our previous results (*6*) and those documented in this study point to (1) a novel regulatory pathway, in which AR-activated EGFR undergoes active sorting to a recycling pathway, (2) a role for c-Cbl in regulating EGFR presorting and recycling, (3) the critical involvement of ESCRT-I and -III in sorting of EGFR destined for recycling, and (4) the possibility that ESCRTs could serve differential functions and/or could be differentially recruited and assembled depending upon EGFR ligand or other variables. In the future, it will be of great interest to determine whether ESCRTs associate with EGFR or whether ESCRTs indirectly affect receptor endocytic sorting. It also will be important to consider the possibility that ESCRT complexes may have differential effects on EGFR recycling depending upon whether they are recycled via sorting or recycling endosomes.

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SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Sorkin, A., and Goh, L. K. (2009) *Exp. Cell Res. 315*, 683–696.
2. Raiborg, C., and Stenmark, H. (2009) *Nature 458*, 445–452.
3. Nickerson, D. P., Russell, M. R., and Odorizzi, G. (2007) *EMBO Rep. 8*, 644–650.
4. Maxfield, F. R., and McGraw, T. E. (2004) *Nat. Rev. Mol. Cell Biol. 5*, 121–132.
5. Hanyaloglu, A. C., and von Zastrow, M. (2008) *Annu. Rev. Pharmacol. Toxicol. 48*, 537–568.
6. Baldys, A., Gooz, M., Morinelli, T. A., Lee, M. H., Raymond, J. R., Jr., Luttrell, L. M., and Raymond, J. R., Sr. (2009) *Biochemistry 48*, 1462–1473.
7. Raiborg, C., Malerod, L., Pedersen, N. M., and Stenmark, H. (2008) *Exp. Cell Res. 314*, 801–813.
8. Huang, F., Goh, L. K., and Sorkin, A. (2007) *Proc. Natl. Acad. Sci. U.S.A. 104*, 16904–16909.
9. Di Fiore, P. P., and Gill, G. N. (1999) *Curr. Opin. Cell Biol. 11*, 483–488.
10. Mosesson, Y., Mills, G. B., and Yarden, Y. (2008) *Nat. Rev. Cancer 8*, 835–850.
11. Ebisuya, M., Kondoh, K., and Nishida, E. (2005) *J. Cell Sci. 118*, 2997–3002.