Internalization and intracellular sorting of the EGF receptor: a model for understanding the mechanisms of receptor trafficking

Inger Helene Madshus[1,2,*] and Espen Stang[1,2]

[1]University of Oslo, Institute of Pathology, Rikshospitalet, N-0027 Oslo, Norway
[2]Division of Pathology, Oslo University Hospital Rikshospitalet, Oslo, Norway
*Author for correspondence (i.h.madshus@medisin.uio.no)

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Summary

The epidermal growth factor receptor (EGFR; also known as ErbB1) is one of four related receptor tyrosine kinases. These receptors (EGFR, ErbB2, ErbB3 and ErbB4) are frequently overexpressed in cancer and such overexpression is associated with poor clinical outcome. Understanding the mechanisms involved in growth-factor-receptor downregulation is medically important, as several drugs that interfere with the function and trafficking of ErbB proteins are currently being developed or are already in clinical trials. EGFR has become a model protein for understanding the biology and endocytosis of related growth-factor receptors, and the mechanisms involved in its endocytosis and degradation have been scrutinized for several decades. Nevertheless, the details and principles of these processes are still poorly understood and often controversial. In particular, the literature describing how the ubiquitylation and recruitment of EGFR to clathrin-coated pits are connected is inconsistent and confusing. In this Opinion article, we discuss the impact of signaling motifs, kinase activity and ubiquitylation on clathrin-dependent endocytosis and lysosomal sorting of EGFR. In addition, we discuss potential explanations for contradicting reports, and propose models for the recruitment of ligand-activated EGFR to clathrin-coated pits as well as for lysosomal sorting of ligand-activated EGFR.

Key words: EGF receptor, Clathrin, Endocytosis, Ubiquitin

Introduction

The epidermal growth factor receptor (EGFR; also known as ErbB1) is so far the best characterized of the four ErbB-family members (EGFR, ErbB2, ErbB3 and ErbB4). EGFR binds to EGF with high affinity in a specific and saturable manner (for a review, see Carpenter and Cohen, 1979). This binding elicits the internalization and intracellular sorting of receptor-ligand complexes and activates growth-modulating signaling pathways. In addition to EGF, other growth factors [such as transforming growth factor-α (TGFα) and amphiregulin] interact with EGFR and, when such interactions are sufficiently stable, EGFR is internalized and eventually degraded in lysosomes. This requires ubiquitin (Ub)-dependent sorting in early endosomes (for a review, see Stuffers et al., 2008).

It has been debated whether ligand-activated EGFR can be endocytosed in a clathrin-independent as well as a clathrin-dependent manner. Sigismund et al. reported that EGFR in HeLa cells was endocytosed via clathrin-coated pits at low concentrations of EGF, but from cholesterol-enriched lipid rafts at high concentrations of EGF (Sigismund et al., 2008; Sigismund et al., 2005). However, our results from experiments with HeLa and HEp2 cells in which expression of clathrin heavy chain was knocked down by small interfering RNA (siRNA) indicate that EGFR is internalized in a clathrin-dependent manner at both high and low concentrations of EGF (Kazazic et al., 2006). Whereas Sigismund et al. reported that ubiquitylation of EGFR took place only at high concentrations of EGF (20 ng/ml) (Sigismund et al., 2005), our results demonstrated that EGFR was in fact ubiquitylated already at 1 ng/ml EGF (Kazazic et al., 2009). These results supported our conclusion that ubiquitylation plays a role in the translocation of EGFR to clathrin-coated pits under physiological conditions (Kazazic et al., 2006; Stang et al., 2004). The observed different concentrations of EGF found to be required to induce ubiquitylation could be due to differing sensitivity of detection by western blotting with ubiquitin-specific antibodies. The discrepancies concerning the effect of cholesterol-extracting or cholesterol-binding reagents on EGFR endocytosis (Kazazic et al., 2006; Sigismund et al., 2005) could further result from methodological differences and from acquired differences in cell clones. It should also be noted that, in our hands, the cholesterol-extracting reagent cyclodextrin was relatively toxic and, importantly, readily inhibited the endocytosis of both the transferrin receptor and EGFR (Kazazic et al., 2006). As noted (Benmerah and Lamaze, 2007), many discrepancies observed for EGFR might be due to experimental differences in methods, cell assays and ligand concentrations. For example, different clones of similar cells could vary.

It should be noted that, using immunoelectron microscopy (immuno-EM), Sigismund et al. detected an EGFR-Ub chimera in caveolae in cells that had not been incubated with EGF. Furthermore, they detected accumulation of wild-type EGFR in caveolae of cells that had been incubated with high concentrations of EGF (Sigismund et al., 2005). Caveolae are specialized and morphologically detectable lipid rafts containing the protein caveolin. Minshall et al. reported that caveolae were mobilized and internalized following activation of the albumin receptor (Minshall et al., 2000). Although our immuno-EM studies also demonstrated that EGFR was present in caveolae, we found the same relative amount of bound EGF localizing to caveolae on incubation with high and low concentrations of EGF, which does not support the idea of rapid recruitment of EGFR to caveolae. Live-cell microscopy further demonstrated that incubating HEp2 cells with high concentrations

of EGF did not increase the mobility of caveolae (Kazazic et al., 2006). In contrast to the role of small lipid rafts, there now seems to be agreement that caveolae are not involved in EGFR internalization (Kazazic et al., 2006; Sigismund et al., 2008). Rather, caveolae seem to be involved in specific transport processes, such as the transcytosis of albumin.

In this Opinion article, we focus on the proteins and mechanisms that are involved in ligand-induced clathrin-dependent endocytosis of EGFR. We also discuss the cues and mechanisms that are involved in degradative sorting versus recycling of EGFR to the plasma membrane. Because ErbB proteins are often overexpressed in cancer cells owing to blocked endocytosis, obtaining more mechanistic understanding and unified models to describe how growth-factor receptors are endocytosed and degraded should help to advance translational cancer research.

Mechanisms of clathrin-dependent EGFR endocytosis

In the following section, we discuss several factors, including adaptor proteins, EGFR kinase activity and ubiquitylation, that have a role in the process of clathrin-dependent EGFR endocytosis.

Clathrin adaptor protein complex 2 (AP-2) in the endocytosis of EGFR

Clathrin triskelia, which are three-legged polymers of clathrin heavy chain, assemble into polyhedral lattice structures on the cytoplasmic surface of the plasma membrane in places of phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] enrichment. Studies in yeast and mammalian cells have demonstrated that PtdIns(4,5)P_2 turnover is required at multiple stages during endocytic-vesicle formation (Abe et al., 2008; Sun et al., 2007). The binding of clathrin to membranes is mediated by adaptor proteins, of which several contain a PtdIns(4,5)P_2-binding domain. The multimeric protein AP-2 is one such adaptor that is crucial for the formation of clathrin-coated pits at the plasma membrane and for the endocytosis of different types of cargo. Knockdown of the expression of the α-subunit or the μ2 subunit of AP-2 with siRNA strongly inhibited the endocytosis of the transferrin receptor (Huang et al., 2004; Johannessen et al., 2006; Motley et al., 2003). However, EGFR endocytosis was inhibited much less by knocking down AP-2 expression than by knocking down the expression of clathrin heavy chain in several studies (Hinrichsen et al., 2003; Johannessen et al., 2006; Motley et al., 2003) and, interestingly, EGF-induced activation of EGFR caused the formation of new clathrin-coated pits in AP-2-depleted cells (Johannessen et al., 2006). This implies that, whereas AP-2 is directly involved in the recruitment of the transferrin receptor to coated pits, it might have a less important function in the recruitment of EGFR, and also that the small residual amounts of AP-2 not silenced by siRNA could be recruited to the plasma membrane upon ligand-induced activation of EGFR.

EGFR, which is endocytosis competent, was demonstrated to bind to AP-2; no binding to AP-2 was observed for ErbB2, ErbB3 and ErbB4 (Baulida et al., 1996; Sorkin and Carpenter, 1993). An adaptor-binding determinant was localized to residues 970-991 of EGFR using in vitro binding assays, and the specificity was confirmed by studies in which competition with a synthetic peptide corresponding to this sequence was shown (Nesterov et al., 1999; Nesterov et al., 1995). It was further demonstrated that Tyr974 in EGFR is part of a YRAL sequence that binds strongly to the μ2 subunit of AP-2 (Sorkin et al., 1996). However, neither mutations in the YRAL motif of EGFR nor overexpression of mutant μ2 significantly inhibited the internalization and downregulation of EGFR (Nesterov et al., 1999; Nesterov et al., 1995; Sorkin et al., 1996), which indicates that the EGFR–μ2-subunit interaction is of minor importance for EGFR endocytosis. This is puzzling, given the fact that EGFR, in contrast to most AP-2-dependent cargo, was demonstrated to co-immunoprecipitate with AP-2 biochemically (Sorkin and Carpenter, 1993). EGFR also contains three NPXY motifs (where 'X' denotes any amino acid), which are analogous to the endocytosis motif of the low-density lipoprotein (LDL) receptor, although none of these motifs were found to be necessary for efficient ligand-induced endocytosis of EGFR (Chang et al., 1993). Furthermore, it was found that a di-leucine motif (Leu110-Leu111) in EGFR was not crucial for the endocytosis of full-length EGFR (Jiang et al., 2003), although it was found to be essential for EGF-induced phosphorylation of the β2 subunit of AP-2 (Huang et al., 2003).

The importance of the interaction between EGFR and AP-2 in the endocytosis of EGFR has thus been constantly debated (Hinrichsen et al., 2003; Johannessen et al., 2006; Motley et al., 2003; Nesterov et al., 1999), and the impact of the binding of AP-2 to tyrosine and di-leucine motifs on the endocytic capacity of EGFR is still unclear (Sorkin, 2004; Wang et al., 2007). A failure to detect large amounts of EGFR in clathrin-coated regions of the plasma membrane in the absence of EGF (Stang et al., 2004; Stang et al., 2000) indicates that EGFR–AP-2 interactions are not constitutively very active. Rather, available information argues that, although AP-2 is important for the formation of clathrin-coated pits, a direct interaction between EGFR and AP-2 is not required for the ligand-induced recruitment of EGFR to coated pits (Johannessen et al., 2006).

EGFR kinase activity in endocytosis

Most data support the notion that EGFR is recruited to clathrin-coated pits only following ligand-induced activation of the EGFR tyrosine kinase (Lamaze and Schmid, 1995). The finding that the recruitment of the adaptor protein growth factor receptor-bound protein 2 (Grb2) and the ubiquitin ligase Casitas B-lineage lymphoma (Cbl) was required and sufficient to induce clathrin-dependent endocytosis of EGFR (Huang and Sorkin, 2005) strongly suggested that the EGFR kinase was required for this process. This is explained by the fact that autophosphorylation of Tyr1068 or Tyr1086 in the tail of EGFR is a prerequisite for the binding of Grb2 to EGFR, and autophosphorylation of Tyr1045 is required for the binding of Cbl (Jiang et al., 2003; Levkowitz et al., 1999). The finding that ligand-induced recruitment to EGFR of a fusion protein containing the Src-homology 2 (SH2) domain of Grb2 and the RING-finger domain of Cbl was sufficient for its clathrin-dependent endocytosis (Huang and Sorkin, 2005) further suggested that EGFR kinase activity and the recruitment of Cbl facilitate the initiation of endocytosis. However, Wang and colleagues showed that receptor dimerization, but not kinase activity, was required for EGFR endocytosis (Wang et al., 2005). This controversial result has thus far been difficult to interpret. It should be noted that cellular stress caused by cytokines and exposure to ultraviolet light has been demonstrated to induce EGFR endocytosis independently of the EGFR tyrosine-kinase activity (Vergarajauregui et al., 2006; Zwang and Yarden, 2006). However, most available data argue that ligand-induced receptor dimerization, with subsequent autophosphorylation of tyrosine residues in the EGFR tail, is required for EGFR endocytosis.

Ubiquitylation of EGFR in endocytosis

Protein ubiquitylation is a post-translational and reversible modification in which a 76-amino-acid polypeptide, Ub, is attached to the ε-amino group of lysine residues in target proteins.

Accumulating information supports the idea that ubiquitylation of receptor tails in mammals triggers endocytosis, similar to the observation that covalently bound Ub drives the endocytosis of transporters and receptors in yeast (Galan et al., 1996; Hicke and Dunn, 2003). Similar to other target proteins (Hershko and Ciechanover, 1998), it has been demonstrated that EGFR can be both monoubiquitylated (one Ub on one lysine) or multiubiquitylated (several monoubiquitylated sites on a protein) and polyubiquitylated (several Ub molecules on one lysine) concurrently (Huang et al., 2006). Monoubiquitylation seems to have a role in intramolecular interactions, whereby Ub binds to Ub-binding domains (UBDs) within the same protein. In general, the monoubiquitylation of Ub-binding proteins has been proposed to inhibit their capacity to bind and control the functions of ubiquitylated targets in vivo by inducing a closed conformation (Hoeller et al., 2006). However, the role of monoubiquitylation of EGFR has thus far not been addressed.

All seven of the lysine residues in Ub (K6, K11, K27, K29, K33, K48 and K63) contribute to the synthesis of polyUb chains on protein substrates. K48-linked chains are mediators of proteasomal degradation, and K63-linked chains act in non-proteolytic events, such as DNA repair and vesicle trafficking (Xu et al., 2009). Modification of target proteins by Ub controls their localization, function and stability by controlling both the intra- and intermolecular interactions (Hurley et al., 2006; Pickart, 2001). Ubiquitylation in the budding yeast *Saccharomyces cerevisiae* was demonstrated early on to direct transmembrane proteins for endocytic uptake (for reviews, see Dupre et al., 2004; Hicke and Dunn, 2003; Staub and Rotin, 2006). In addition, Ub acts as an endocytosis signal in both yeast and mammalian cells by interacting with UBDs in endocytic adaptor proteins. There is an expanding group of UBDs (for a review, see Hurley et al., 2006).

Interestingly, the Ub-interaction motif (UIM)-containing proteins Eps15 and epsin-1 (Hofmann and Falquet, 2001) were observed to localize to clathrin-coated pits (Edeling et al., 2006; Stang et al., 2004; Stang et al., 2000; Tebar et al., 1996), from which EGFR is internalized (Carpentier et al., 1982; Kazazic et al., 2006). Indeed, we recently observed that siRNA-mediated knockdown of epsin-1 expression modestly inhibited clathrin-dependent endocytosis of ubiquitylated EGFR. Consistently, EGFR was inefficiently recruited into the invaginated part of clathrin-coated pits in cells in which epsin-1 expression was knocked down (Kazazic et al., 2009). This argues that epsin-1 is involved in the translocation of ubiquitylated EGFR from the smooth plasma membrane to clathrin-coated areas, and thereby promotes endocytosis (Fig. 1). The finding that knocking down epsin-1 expression has only a modest effect indicates that other Ub-binding proteins are probably also required for this process. Other Ub-binding proteins could function in parallel with epsin-1, one of which might be CIN85, which was recently demonstrated to bind to Ub via its SH3 domains (Bezsonova et al., 2008). Apparently, disruption of the capacity of CIN85 to bind to Ub resulted in constitutive ubiquitylation of CIN85 and increased levels of EGFR ubiquitylation in the absence of EGFR-ligand binding. The SH3 domains of CIN85 also bind to the Ub ligase Cbl (Take et al., 2000), which ubiquitylates EGFR (Levkowitz et al., 1999; Levkowitz et al., 1998). This suggests that competitive binding of Cbl and Ub to CIN85 could regulate EGFR endocytosis (Bezsonova et al., 2008).

Ubiquitylation of EGFR depends on the Ub ligases c-Cbl and Cbl-b, and recently, Ubc4/5 was identified as the E2 Ub-conjugating enzyme that cooperates with c-Cbl (Umebayashi et al., 2008). Combined siRNA-mediated knockdown of c-Cbl and Cbl-b expression was found to inhibit the internalization of ^{125}I-labeled EGF (Huang et al., 2007; Huang et al., 2006). This finding

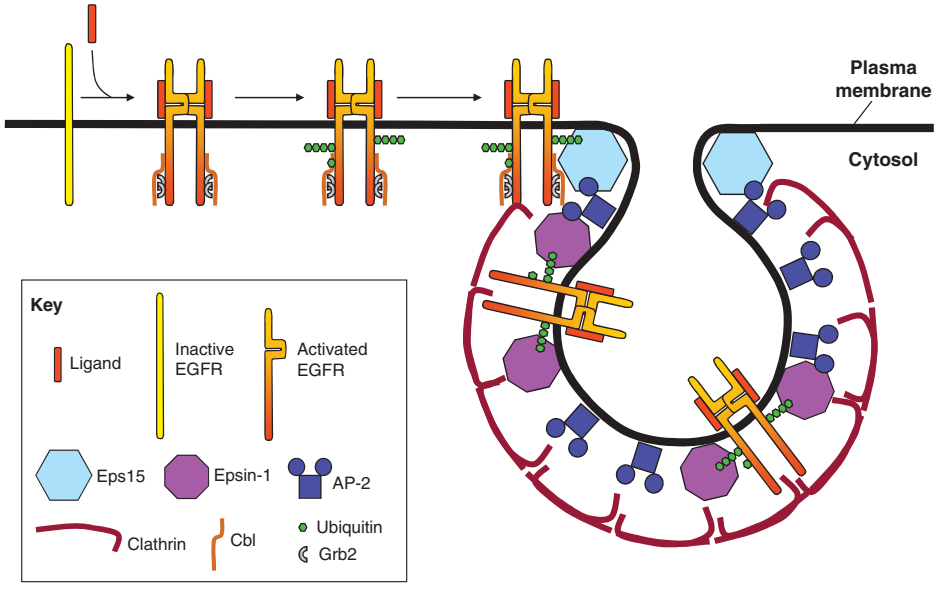

Fig. 1. Current model illustrating the events required for recruitment of EGFR into clathrin-coated pits. We propose the following scenario for ligand-induced clathrin-dependent EGFR endocytosis: ligand binding to EGFR induces receptor dimerization and kinase activity. Kinase activity results in the phosphorylation of tyrosines in the EGFR tail, allowing Cbl to bind directly, or indirectly through Grb2. Recruitment of Cbl results in mono- and polyubiquitylation of the EGFR tail. Appended polyUb chains interact with Ub-interaction motifs (UIMs) of epsin-1 and Eps15. Because Eps15 is localized at the rim of clathrin-coated pits (Stang et al., 2004; Tebar et al., 1996), we propose that ubiquitylated EGFR initially interacts with Eps15. In contrast to Eps15, epsin-1 is localized all along the clathrin coat; we further propose that EGFR is transferred to epsin-1 and thereby recruited into the central region of clathrin-coated pits (Kazazic et al., 2009). The clathrin-coated membrane subsequently invaginates and is released into the cell interior as a clathrin-coated vesicle.

suggested that Cbl is required for clathrin-dependent EGFR internalization, and is consistent with previous reports that Cbl mutants had dominant-negative effects on EGFR internalization (Jiang and Sorkin, 2003) and with the finding that a Cbl-Grb2 chimeric protein containing the RING-finger domain of Cbl rescued EGFR endocytosis in Grb2-depleted cells (Jiang et al., 2003). We previously demonstrated that activated EGFR was ubiquitylated at the plasma membrane in HeLa cells that were, owing to overexpression of mutant dynamin 1, endocytosis deficient (Stang et al., 2000). The fact that ubiquitylation of EGFR preceded clathrin-mediated endocytosis of EGFR suggested that Ub could be a cue for the translocation of EGFR to clathrin-coated pits. Consistently, the overexpression of either human sprouty homolog 2 (Spry2; which binds to Cbl and thus inhibits ligand-induced ubiquitylation of EGFR) or overexpression of a mutant form of Ub lacking the C-terminal Gly residue (which is required for conjugation of Ub) blocked recruitment of EGFR into clathrin-coated pits, but did not inhibit endocytosis of the transferrin receptor (Stang et al., 2004). These results further support the notion that ubiquitylation of EGFR is involved in its recruitment to clathrin-coated pits.

Mass-spectrometric analysis has revealed that EGFR is both polyubiquitylated and multiubiquitylated within 5 minutes of EGF addition in vitro, and that a large proportion of the Ub moieties in polyUb chains are linked through Lys63 (Huang et al., 2006). Although it has been more or less established that ubiquitylation controls clathrin-dependent endocytosis of several mammalian receptors and transporters, such as the leptin receptor OB-Ra (Belouzard and Rouille, 2006), the glutamate transporter (GLT1) (Gonzalez-Gonzalez et al., 2008), the dopamine receptor (Miranda et al., 2007) and the nerve-growth-factor receptor TrkA (Geetha et al., 2005), it is still debated whether ubiquitylation of EGFR is necessary for EGFR endocytosis. On the basis of findings that EGFR mutants that are ubiquitylated inefficiently [Y1045F-EGFR (which is incapable of directly binding Cbl via phosphorylated Tyr1045), 9KR-EGFR and 15KR-EGFR] were efficiently endocytosed, it was concluded that EGFR ubiquitylation was not necessary for its internalization (Huang et al., 2007). However, it was also reported that the endocytosis of an EGFR mutant with impaired kinase activity was rescued when two main ubiquitylation sites were reconstituted (Huang et al., 2007). These results suggested that ubiquitylation is required for EGFR internalization, at least under conditions of inefficient EGFR kinase activity. It should be noted that, under all conditions, endocytosis of EGFR mutants was dependent on functional Grb2, Cbl and clathrin (Huang et al., 2007).

When Ub was recombinantly added to the C-terminal region of a deletion mutant of EGFR lacking the intracellular domain (and thus lacking both the kinase domain and the tyrosines in the EGFR tail), internalization of EGF was observed (Sigismund et al., 2005). This indicates that Ub can act as an endocytosis signal. Our results suggest that ubiquitylation induces the translocation of EGFR to clathrin-coated pits (Stang et al., 2004), but those of Sigismund et al. indicate that ubiquitylated EGFR localizes to lipid rafts (Sigismund et al., 2005). The reasons for this discrepancy have not been clarified (see above).

Barriere et al. presented evidence that only multiple monoUb moieties or polyUb chains are recognized by the endocytic machinery in vivo and can associate with a subset of Ub-binding clathrin adaptors in vitro (Barriere et al., 2006). Genetic and pharmacological approaches demonstrated that internalization of plasma-membrane proteins with multiple Ub moieties was clathrin dependent but caveolin independent (Barriere et al., 2006). The finding that multiple interactions between Ub and the UIMs of the target proteins are required is explained by the low affinity of monoUb for the UIM (see also Hawryluk et al., 2006).

To determine whether ubiquitylation of EGFR is sufficient for clathrin-dependent EGFR endocytosis, it would be necessary to compare the internalization of wild-type EGFR with that of EGFR mutants that had normal kinase activity but that could not be ubiquitylated. However, it has thus far not been possible to completely isolate the effects of ubiquitylation from those of kinase activity in EGFR mutants (Huang et al., 2007). As recently observed (Kazazic et al., 2009), small amounts of Ub conjugated to EGF-bound EGFR seemed to be sufficient both for mediating UIM-dependent binding of epsin-1 to EGFR and initiating endocytosis. This was demonstrated by comparing the efficiency with which the epsin N-terminal homology (ENTH)-UIM domain of epsin-1 co-immunoprecipitated with either wild-type EGFR or a Y1045F-EGFR mutant. These EGFRs were found to co-immunoprecipitate equally efficiently, although the ubiquitylation of Y1045F-EGFR was strongly reduced compared with that of wild-type EGFR (Kazazic et al., 2009). The finding that wild-type EGFR and Y1045F-EGFR are internalized with the same efficiency has been previously demonstrated (Jiang et al., 2003; Jiang and Sorkin, 2003). Additionally, the ubiquitylation of EGFRs that contained multiple point mutations (in which lysines were converted to arginines) was strongly reduced compared with wild type, although the endocytosis of these mutant EGFRs was not inhibited (Huang et al., 2007).

The lack of correlation when the extent of ubiquitylation and rate of internalization of EGFR were compared was interpreted to mean that ubiquitylation is not required for internalization. However, we favor the alternative explanation that small amounts of Ub conjugated to EGFR are sufficient for rapid and saturable endocytosis. The findings that support this idea are numerous: it was shown that both wild-type EGFR and Y1045F-EGFR contained K63-linked polyUb chains even though the overall ubiquitylation of Y1045F-EGFR was strongly reduced (Huang et al., 2006). Interestingly, K63- and K48-linked polyUb chains have been demonstrated to be involved in efficient binding to the UIM of epsin-1 (Hawryluk et al., 2006). This could explain the efficient EGFR–epsin-1 interaction and therefore the efficient endocytosis of Y1045F-EGFR (Kazazic et al., 2009). The length of the polyUb chains that is sufficient to anchor the EGFR to epsin-1 is unclear. In addition, it is not known whether there are other as-yet-unidentified Ub-binding proteins that are involved in the recruitment of EGFR to clathrin-coated pits. Monoubiquitylation probably contributes less to UIM binding than do multi- and polyubiquitylation, unless increased avidity for mono- or di-ubiquitylated residues occurs owing to the close proximity induced by receptor oligomerization (Barriere et al., 2006). It should be noted that several methodological challenges probably still need attention and consideration to obtain accurate quantification of ubiquitylation. For example, antibody-based estimation of ubiquitylation levels by western blotting is technically difficult and often depends on the total amounts of loaded protein (Umebayashi et al., 2008).

Mechanisms controlling degradation versus recycling of EGFR

Internalized receptors are delivered to a tubulovesicular compartment known as the early or sorting endosome. From here,

receptors can be recycled back to the plasma membrane or selected for lysosomal sorting by incorporation into small vesicles that bud away from the limiting membrane into the lumen of multivesicular bodies (MVBs). Lysosomal sorting is important for negatively regulating proliferative and anti-apoptotic signaling mediated by EGFR, because it segregates the kinase from its cytosolic substrates and induces the degradation and downregulation of EGFR expression. Important pioneering experiments in yeast demonstrated that both receptor internalization and proteolysis in the vacuole were strongly impaired in cells that were defective for ubiquitylation and deubiquitylation enzymes. The same result was observed in cells expressing receptors and channels that contained mutations in ubiquitylation sites (Hicke and Riezman, 1996; Rotin et al., 2000). Furthermore, receptors and transporters were found to accumulate at the plasma membrane in their ubiquitylated form in endocytosis-defective cells (Kolling and Hollenberg, 1994). This argued that ubiquitylation and deubiquitylation are required both for endocytosis and for degradation in the vacuole.

As discussed above, the requirement for ubiquitylation in clathrin-dependent endocytosis of EGFR is still debated. By contrast, the requirement of ubiquitylation for lysosomal degradation of EGFR was previously agreed on, although it is still unclear whether mono- versus polyUb, or specific polyUb linkages, are required. We previously demonstrated that EGFR was ubiquitylated while at the plasma membrane (Stang et al., 2000), but that EGFR seemed to be continuously deubiquitylated and reubiquitylated en route to the lysosome (Longva et al., 2002). EGF, which remained stably associated with EGFR in the acidic environment of endosomes, maintained EGFR kinase activity, ubiquitylation and degradation. However, the ligand TGFα, which dissociates from EGFR at low pH, induced only transient EGFR ubiquitylation and thereby induced EGFR recycling instead of degradation (Longva et al., 2002). Furthermore, the Y1045F-EGFR mutant, which only binds Cbl indirectly via Grb2, is poorly ubiquitylated and is rapidly recycled following endocytosis (Grovdal et al., 2004; Jiang and Sorkin, 2003). This argues that, although ubiquitylation is a signal for both endocytosis and lysosomal sorting, the ubiquitylation patterns required to trigger these trafficking events are different.

The requirement for Ub in degradative sorting is explained by the nature of the sorting machinery, which is composed of four well-characterized complexes that make up the endosomal sorting complex required for transport (ESCRT) machinery. Three of the ESCRT complexes contain Ub-binding subunits (Stuffers et al., 2008). In addition, deubiquitylating enzymes (DUBs) might function at the endosome to oppose Ub E3 ligase activity or to recycle Ub from receptors that have been committed to the sorting pathway. AMSH and UBPY (also known as Usp8) are endosomally localized DUBs that have been reported to affect the sorting of endocytosed EGFR (reviewed by Clague and Urbe, 2006). AMSH interacts with the SH3 domain of STAM (McCullough et al., 2004) and with Hrs (which are both components of ESCRT-0). It has been suggested that AMSH deubiquitylates EGFR and thereby rescues it from

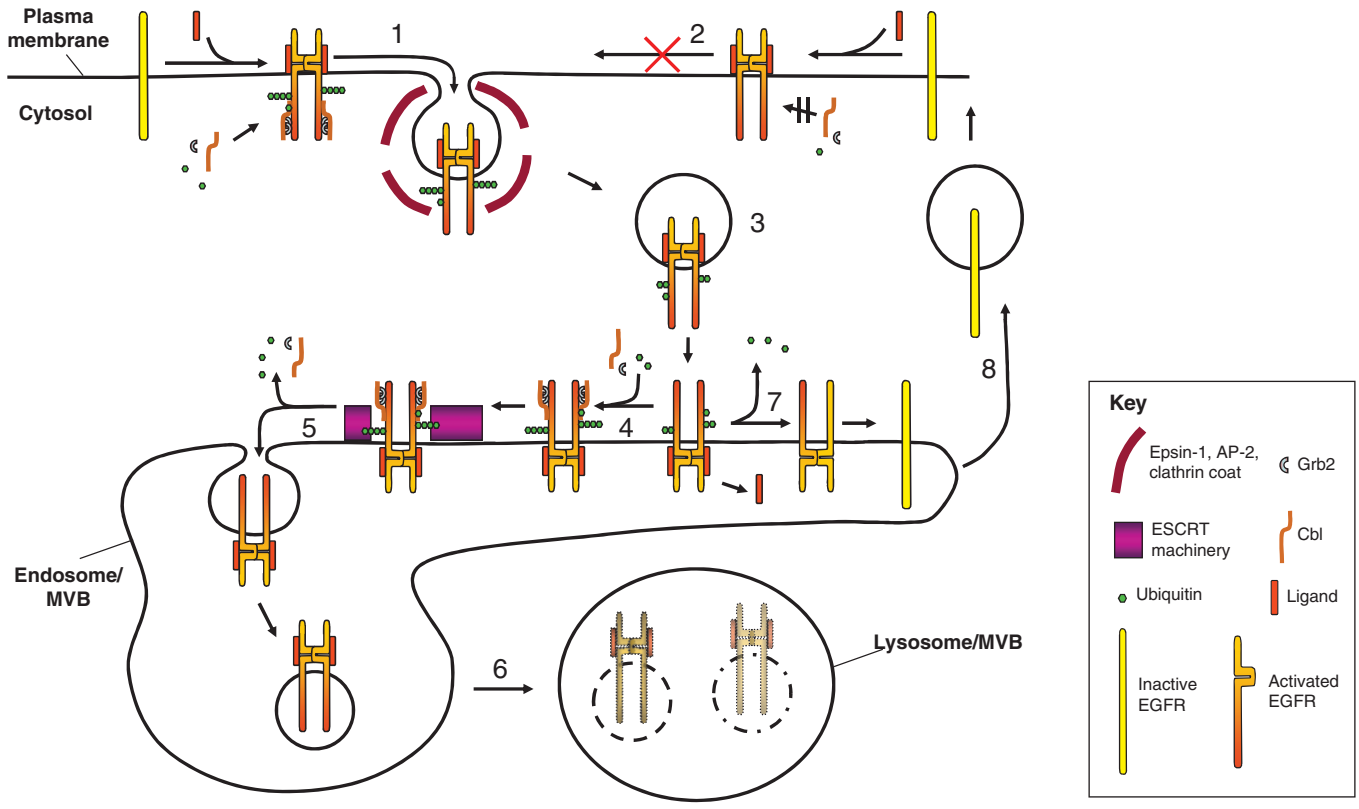

Fig. 2. Current model illustrating endosomal trafficking of EGFR. On ligand binding, EGFR is activated, ubiquitylated and recruited into clathrin-coated pits (1). However, when ubiquitylation of activated EGFR is blocked, its recruitment into coated pits is inhibited (2). We propose that EGFR is partially deubiquitylated following internalization (3), but that ligand-bound EGFR is reubiquitylated (Longva et al., 2002) (4). Through interaction with the ESCRT machinery on early endosomes, EGFR is sorted to inner vesicles of endosomes (5) and finally to MVBs and lysosomes for degradation (6). If, however, the EGFR ligand dissociates at low pH, EGFR that localizes to endosomes is deactivated and deubiquitylated (7) and recycled (8) to the plasma membrane.

lysosomal degradation. The role of UBPY, which also binds to the SH3 domain of STAM (Kato et al., 2000), is less clear, and different effects of siRNA-mediated knockdown of UBPY expression on EGFR sorting have been reported (Clague and Urbe, 2006). Although in vitro DUB assays demonstrated strong selectivity of AMSH for K63-linked versus K48-linked chains, UBPY was also demonstrated to trim and remove both K48-linked and K63-linked chains from target proteins (McCullough et al., 2006; Mizuno et al., 2005). It is possible that different DUBs collaborate with Ub ligases to remodel Ub chains. Such remodeling of Ub chains has been reported in yeast to occur in proteasomes through the opposing effects of the Ub ligase Hul5 and the DUB Ubp6 (Crosas et al., 2006). Because the activated EGFR was reported to contain both K48-linked and K63-linked polyUb chains (Huang et al., 2006), it is tempting to speculate that proteasomal activity could be involved in degradative lysosomal sorting of EGFR. Indeed, the proteasome inhibitor lactacystin was found to inhibit degradation of EGFR (Longva et al., 2002). However, immuno-EM experiments demonstrated that EGFR that was localized to intralumenal endocytic vesicles was recognized by antibodies specific for both intra- and extracellular epitopes, arguing that EGFR itself is not targeted for proteasomal degradation (Longva et al., 2002). Interestingly, the morphology of MVBs was altered following incubation with proteasome inhibitors, and the formation of intralumenal vesicles was inhibited when cells were incubated with the proteasome inhibitor lactacystin and EGF. This suggests that proteasome activity is required for the formation of EGFR-containing MVBs, and future studies might identify proteasomal substrates with a role in lysosomal sorting.

Conclusions and perspectives

EGFR is endocytosed and sorted to lysosomes upon ligand-induced activation. It is controversial as to what extent endocytosis of EGFR is clathrin-dependent upon ubiquitylation. Although it is mostly accepted that ubiquitylation is a required cue for lysosomal sorting of EGFR, the role of ubiquitylation in endocytosis is still a debated issue. We propose the following model for endocytosis and lysosomal sorting of ligand-bound EGFR: first, activation of the EGFR kinase induces phosphorylation of tyrosines, of which Tyr1045-*P*, Tyr1068-*P* and Tyr1086-*P* are required for direct or indirect interaction with the ubiquitin ligase Cbl. The resulting ubiquitylation is important for the recruitment of EGFR to clathrin-coated pits through interaction with Ub-binding adaptor proteins, such as Eps15 and epsin-1 (see Fig. 1). Second, stable ligand binding secures kinase activity and ubiquitylation, which is required to avoid recycling of EGFR to the plasma membrane (see Fig. 2). Upon sequential interaction with the ESCRT machinery at the limiting membrane of early endosomes, EGFR is eventually deubiquitylated and translocated to inner vesicles of endosomes by poorly understood mechanisms. Once in these inner vesicles, EGFR is destined for lysosomal degradation.

It cannot be excluded that the activated and ubiquitylated EGFR is endocytosed clathrin-independently under certain conditions. It should, however, be noted that even small amounts of conjugated Ub seem to be sufficient for ligand-activated EGFR to interact with Ub-binding proteins such as epsin-1 (Kazazic et al., 2009). We therefore conclude that endocytosis of EGFR is Ub guided. Future studies should determine the extent and patterns of ubiquitylation that are required for endocytosis and lysosomal sorting. Much of the existing controversy can probably be explained by the crude and suboptimal methods that are currently available for characterizing ubiquitylation. In most studies carried out so far, mass spectrometry or western blotting with Ub-specific antibodies was used and, as mentioned above, the antibodies might in many cases be ill-suited for careful characterization of patterns and amounts of ubiquitylation. In addition, it is now known that Ub chains are trimmed as proteins move from the cell surface to their final intracellular destination. Elucidating how Ub ligases collaborate with DUBs and, potentially, with proteasomes in such processes will require new techniques and reagents that allow quantitative and qualitative characterization. Ideally, techniques that allow dynamic reading of such processes should be developed.

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