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Endocytosis of Receptor Tyrosine Kinases

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Endocytosis is the major regulator of signaling from receptor tyrosine kinases (RTKs). The canonical model of RTK endocytosis involves rapid internalization of an RTK activated by ligand binding at the cell surface and subsequent sorting of internalized ligand-RTK complexes to lysosomes for degradation. Activation of the intrinsic tyrosine kinase activity of RTKs results in autophosphorylation, which is mechanistically coupled to the recruitment of adaptor proteins and conjugation of ubiquitin to RTKs. Ubiquitination serves to mediate interactions of RTKs with sorting machineries both at the cell surface and on endosomes. The pathways and kinetics of RTK endocytic trafficking, molecular mechanisms underlying sorting processes, and examples of deviations from the standard trafficking itinerary in the RTK family are discussed in this work.

Functional activities of transmembrane proteins, including the large family of RTKs, are controlled by intracellular trafficking. RTKs are synthesized in the endoplasmic reticulum, transported to Golgi apparatus, and then delivered to the plasma membrane. At the cell surface RTKs undergo constitutive endocytosis (internalization) at a rate similar to that of other integral membrane proteins. Constitutive internalization of RTKs is much slower than their constitutive recycling from endosomes back to the cell surface. Therefore, RTKs are accumulated at the cell surface, which allows maximal accessibility to extracellular ligands. The rates of the constitutive internalization, recycling, and degradation determine the half-life of an RTK protein, which varies depending on the nature of the RTK and the cell type, and typically positively correlates with the expression level of the

RTK. For example, the turnover rates range from $t_{1/2} < 1$ h for the colony stimulating factor 1 receptor (CSF-1R) in macrophages (Lee et al. 1999) to 24 h for the epidermal growth factor receptor (EGFR) overexpressed in carcinoma cells (Stoscheck and Carpenter 1984).

Activation of RTKs by growth factors elevates turnover and ultimately leads to down-regulation of RTKs. First, at the cell surface, ligand binding increases the rate of RTK internalization by several folds (Fig. 1). Typically, surface RTKs can freely diffuse in the plane of the plasma membrane and, therefore, reach all endocytic sites. In some cases, however, a pool of RTKs is immobile owing to association with actin-based membrane protrusions (microvilli) or caveolae (Mineo et al. 1999; Foti et al. 2004; Hommelgaard et al. 2004). This pool is incapable of rapid internalization and retained at the

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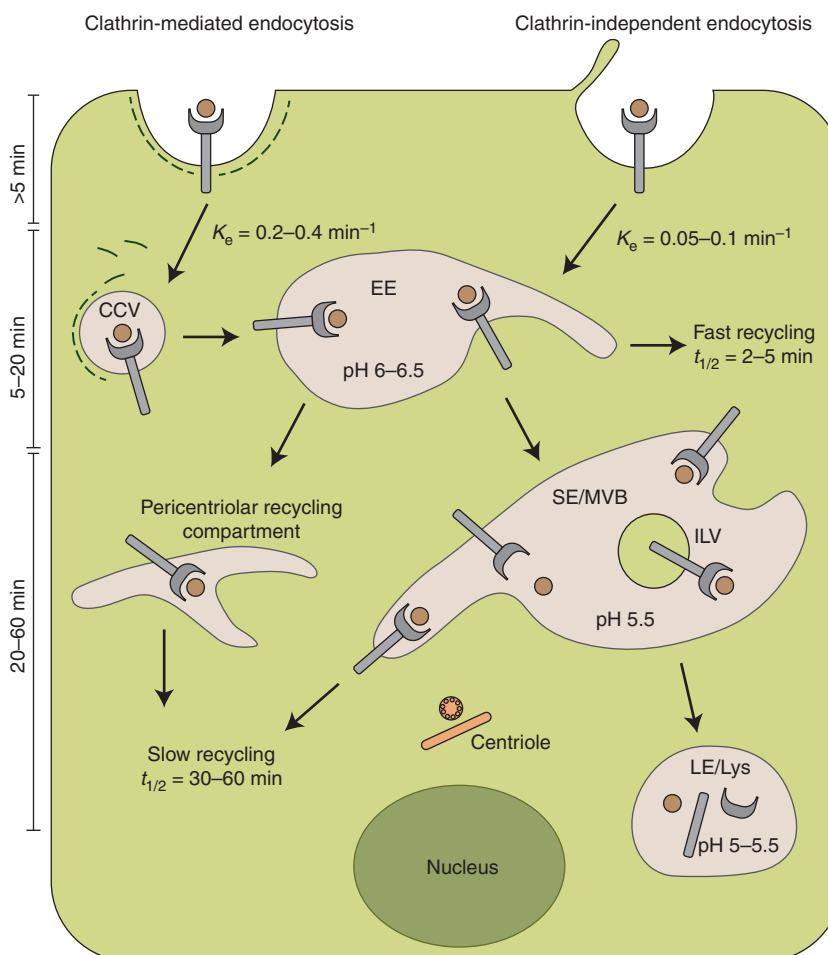


Figure 1. Pathways of RTK endocytosis. RTKs are endocytosed by clathrin-mediated and clathrin-independent mechanisms. Typical rate constants (K_e) for RTK internalization through both pathways are shown. Clathrin-coated vesicles are uncoated shortly after fission from the plasma membrane and fuse with early endosomes (EEs). In the most well-studied EGFR system, **EGFR-ligand complexes are detected in these highly dynamic, morphologically heterogeneous compartments within 2–5 min after EGF stimulation** (Haigler et al. 1979; Beguinot et al. 1984; Miller et al. 1986; Hopkins et al. 1990). Ligand-RTK complexes remain intact but certain ligands dissociate from the receptor in the acidic environment of the endosomal lumen. Released ligands remain in the vesicular parts of endosomes (most of the endosomal volume), whereas unoccupied receptors are found mainly in tubular extensions (most of the membrane area). Ligand-occupied and unoccupied RTKs can rapidly recycle from EEs through the process of back fusion of peripheral EEs with the plasma membrane or via tubular carriers derived from these endosomes (retroendocytosis). EEs mature into sorting endosomes (SE) or multivesicular bodies (MVBs) in which RTKs are incorporated into intraluminal vesicles (ILVs) by inward membrane invagination. RTKs can also be delivered to the pericentriolar Rab11-containing recycling compartment. Recycling of unoccupied and ligand-occupied RTKs is slower from the SE/MVBs and recycling compartment. SE/MVBs gradually lose early endosome components, such as Rab5 and EEA1, and recycling cargo (such as transferrin receptors) while become enriched in resident late endosomal proteins (such as Rab7), thus maturing into late endosomes. Fusion of late endosomes with primary lysosomes carrying proteolytic enzymes results in degradation of receptors and growth factors. A typical time scale of RTK endocytosis, their accumulation in EEs, and SE/MVBs is shown in minutes.



cell surface. EGFR and its closest homolog, ErbB2, can also be retained in the plasma membrane through interactions with Na^+/K^+ exchanger and erbin, respectively (Borg et al. 2000; Lazar et al. 2004). Thus, interplay of surface retention and endocytosis-promoting mechanisms determines the extent of activation-induced acceleration of RTK internalization. The outcomes of this interplay vary within the RTK family. For instance, endocytosis of ErbB2 is not significantly increased when ErbB2 is activated by homo- or heterodimerization with other ErbBs (Yarden and Sliwkowski 2001). In contrast, some RTKs display a relatively fast constitutive internalization that is not appreciably augmented by ligand binding (McClain 1992; Burke and Wiley 1999; Jopling et al. 2011).

The second trafficking step causing down-regulation of activated RTKs is the efficient sorting of internalized RTKs to late endosomes and lysosomes for proteolytic degradation (Fig. 1). Members of all RTK subfamilies studied to date undergo accelerated lysosomal degradation on activation. Thus, activity-dependent acceleration of both sorting processes, at the cell surface and in endosomes, together resulting in RTK down-regulation, is a trademark of this receptor family. Significant advances have been made in recent years in understanding the molecular mechanisms of RTK endocytic trafficking. Yet, the specific components that mediate and regulate key internalization and sorting processes remain poorly defined. Because many conceptual findings have been made using the EGFR system and later reproduced in studies of other RTKs, EGFR has become the prototypic model system to study RTK endocytosis. Thus, we will rely on data derived in EGFR models for the broader discussion of other RTK families.

Endocytosis is the major regulator of signal transduction processes initiated by RTKs. Although the discussion of cross talk between endocytosis and signaling is outside of the scope of this article, we should emphasize that elucidation of the specific mechanisms of RTK endocytosis is essential for defining the role of endocytosis in regulation of RTK signaling. Hence our discussion will focus on various mo-

lecular mechanisms implicated in RTK endocytic trafficking. We regret that we are unable to cite many excellent studies on RTK endocytosis owing to the space constraints. We would also like to point out that a major limitation in the interpretation of RTK endocytosis literature is that methods used to analyze RTK internalization in the majority of studies fail to measure specific internalization rates because interference of receptor recycling is rarely considered. Therefore, changes in the apparent rates of RTK endocytosis observed in such studies could be owing to alterations in either internalization or recycling. We will discuss the literature in accordance to the authors' interpretation, but leave the detailed evaluation of each set of published data to reader discretion.

PATHWAYS OF RTK INTERNALIZATION

On ligand activation, increased localization of EGFR, neurotrophic tyrosine kinase receptor type 1 (TrkA), insulin receptor (IR), and a number of other RTKs within clathrin-coated pits (CCPs) has been observed (Gorden et al. 1978; Carpentier et al. 1982; Beattie et al. 2000; Bogdanovic et al. 2009). Further, internalization of EGFR and several other RTKs was blocked by small interfering RNAs (siRNAs) to clathrin heavy chain and chemical inhibitors of clathrin (Huang et al. 2004; Lampugnani et al. 2006; Zheng et al. 2008; von Kleist et al. 2011). Together, these studies show that clathrin-mediated endocytosis (CME) is the major pathway of internalization for ligand-occupied RTKs (Fig. 1).

CME is the fastest internalization pathway (rate constant $K_e \sim$ up to 0.6 min^{-1}). Kinetics analysis of EGFR endocytosis suggested that CME is saturated when a large number of surface EGFRs are activated by EGF, and the contribution of a slower clathrin-independent endocytosis (CIE) increases with the increase of EGF concentration and EGFR expression levels, leading to overall reduction in the apparent internalization rate (Wiley 1988). Indeed, siRNA depletion of clathrin significantly inhibited EGFR internalization only when low concentrations of EGF were used (Sigismund et al. 2005). Similar saturation of the rapid internalization

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pathway and reduced rates of internalization were observed when IR and insulin-like growth factor 1 receptor (IGF-1R) were activated with high ligand concentrations (Backer et al. 1991; Prager et al. 1994). CIE has also been shown for fibroblast growth factor receptor (FGFR) and vascular endothelial growth factor receptor (VEGFR) (Wiedlocha and Sorensen 2004; Lannan et al. 2010; Haugsten et al. 2011).

Mechanisms of CIE for various RTK can be roughly divided into two groups. The macropinocytosis-like endocytosis involving actin cytoskeleton rearrangements and membrane ruffling has been observed in early studies of epidermoid carcinoma A-431 cells that express very high levels of EGFR (Haigler et al. 1979), and later in other cell types (Barbieri et al. 2000; Yamazaki et al. 2002; Orth et al. 2006; Valdez et al. 2007). Another mechanism implicated in the CIE of RTKs is defined by its sensitivity to inhibitors of caveolae and cholesterol-disrupting agents (Sigismund et al. 2005; Sehat et al. 2008; Salani et al. 2010). However, the lack of experimental tools to inhibit specific CIE pathways impedes analysis of the mechanisms and functional role of CIE. Given the major role of CME in the presence of physiological concentrations of growth factors, we therefore focus our discussion on the molecular mechanisms of this pathway for RTK endocytosis.

ROLE OF TYROSINE-BASED AND DILEUCINE MOTIFS IN RTK ENDOCYTOSIS

Sequence motifs in the cytoplasmic domains of transmembrane proteins are recognized by components of the clathrin coat to trap the endocytic cargo in CCP. Several such motifs have been implicated in RTK internalization (Fig. 2). IR uses the dileucine D(E)xxxLL(I) ("LL") internalization motif (Haft et al. 1994; Morrison et al. 1996; Hamer et al. 1997), which directly binds to the trunk domain of the clathrin adaptor protein complex AP-2 (Fig. 3). The NPxY motif (x, any amino acid residue) was shown to mediate internalization of IR, IGF-1R, and platelet-derived growth factor receptor (PDGFR) β (Chen et al. 1990; Backer et al. 1992; Prager et al. 1994; Wu et al. 2003a). NPxY motifs

interact with the phosphotyrosine-binding domains present in several monomeric adaptor proteins that, in turn, bind to the appendage domains of AP-2 and the amino-terminal domain of clathrin (Fig. 3) (Traub 2009).

Despite the importance of LL and NPxY motifs for internalization of IR and IGF-1R, the interaction of these receptors with AP-2 has not been shown. Such interaction was shown only for EGFR (Sorkin and Carpenter 1993). The YRAL motif of EGFR is responsible for binding to the μ 2 subunit of AP-2 (Sorkin et al. 1996). However, mutation of this motif or inactivation of the tyrosine-binding interface of μ 2 did not affect EGFR internalization (Sorkin et al. 1996; Nesterov et al. 1999). The LL motif in EGFR is important for phosphorylation of Tyr6 in the β 2 subunit of AP-2, suggesting the role of this LL motif in EGFR:AP-2 interaction (Huang et al. 2003). Interestingly, tyrosine phosphorylation of β 2 Tyr6 was predicted to facilitate LL motif interaction with AP-2 (Kelly et al. 2008). However, mutation of this motif also did not affect clathrin-dependent EGFR internalization. Overall, the data on the role of internalization motifs and AP-2 in RTK internalization are incomplete, and the contribution of RTK:AP-2 interactions in ligand-induced RTK endocytosis remains to be defined.

MECHANISMS AND ROLE OF RTK UBIQUITINATION IN CME: CBL AND GRB2

Growth factor binding to RTKs leads to activation of the tyrosine kinase and autophosphorylation of tyrosine residues in the cytoplasmic domain of RTKs, which serve as interaction sites for proteins mediating RTK ubiquitination, a posttranslational modification by the covalent attachment of the ubiquitin polypeptide to lysine residues (Fig. 2). Ubiquitination has historically been considered as the modification that targets proteins for proteosomal degradation, but more recently the role of ubiquitin as the sorting signal in membrane trafficking has emerged (Acconcia et al. 2009). PDGFR- β and EGFR were the first RTKs found to be ubiquitinated (Mori et al. 1992; Galcheva-Gargova et al. 1995). Later, ubiquitination of all

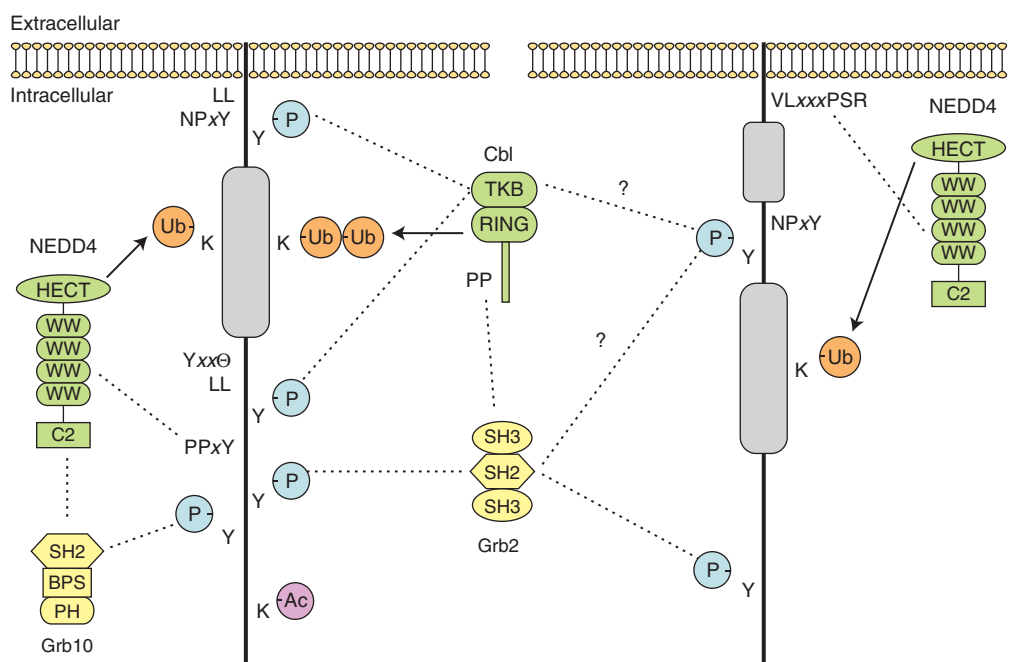


Figure 2. Sequence motifs and posttranslational modifications of RTKs involved in endocytosis. Examples of RTKs with a single kinase domain (EGFR, Met, IR/IGF-1R, Trk subtypes) and an insert-divided kinase domain (PDGFR, FGFR, kit, CSFR, VEGFR) are shown. Sequence motifs containing phosphorylated tyrosine residues (P-Y and Y-P) serve as binding sites for SH2 domains of Grb2, Grb10, and the tyrosine kinase binding (TKB) domain of c-Cbl, Cbl-b, and Cbl-3. Proline-rich motifs in Cbl-b and c-Cbl bind to SH3 domains in Grb2. WW domains of NEDD family E3 ligases bind to PPxY motifs in RTKs, and in case of FGFR, to an unconventional motif in the juxtamembrane domain of this receptor. C2 domain of NEDD4-1 binds to the Grb10 SH2 domain that also binds to phosphotyrosine-containing motifs. Lysine residues are conjugated to ubiquitin in the kinase domains by Cbl, NEDD4, and other E3 ligases. Acetylation of distal lysines in the carboxyl terminus of EGFR is shown. Internalization motifs NPxY, YxxΘ (Θ, bulky hydrophobic residue), and LL are located in juxtamembrane domains, kinase insert, and carboxy-terminal tails of receptors.

major subtypes of RTKs has been shown; thus ubiquitination is a characteristic modification of this receptor family.

The leading model of RTK endocytosis presumes that ubiquitinated RTKs are recruited into CCP by interacting with epsin, Eps15, and Eps15R, proteins that contain ubiquitin-binding domains (UBDs) and bind to clathrin or AP-2 (Fig. 3). However, direct evidence to support this model is still lacking for most RTKs. Ubiquitin-conjugation sites were mapped in EGFR and IGF-1R, and mutational analysis has been performed to assess the importance of ubiquitination sites for the internalization of EGFR, IGF-1R, and FGFR-2 (Huang et al. 2006; Haugsten et al. 2008; Mao et al. 2011). These studies

revealed that ubiquitination of EGFR and FGFR-2 is not essential for internalization. In contrast, mutation of lysines in the IGF-1R significantly decreased antibody-induced endosomal accumulation of this receptor (Mao et al. 2011).

The indirect evidence supporting the role of ubiquitination in RTK endocytosis is based on the effects of mutations or down-regulation of the components of the ubiquitination systems. Ubiquitination is mediated by the sequential action of the ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes (Pickart 2004). E3 ligases determine the substrate specificity of ubiquitination. One large family of E3s that ubiquitinate RTKs is the RING finger containing ubiquitin ligases, such

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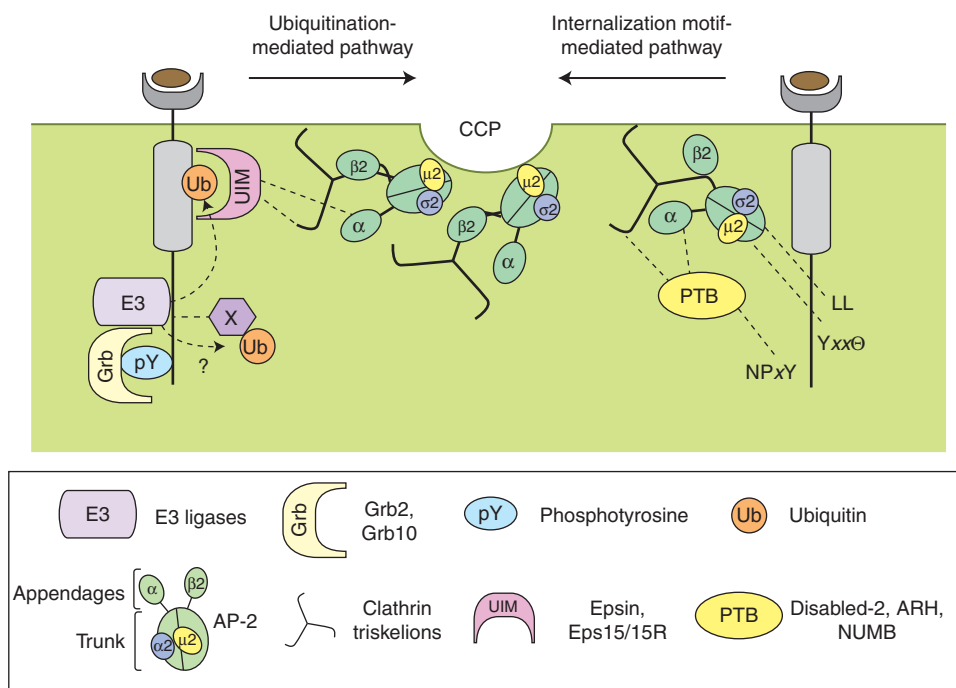


Figure 3. Hypothetic model of clathrin-mediated internalization of ligand-activated RTK. On ligand binding, RTKs are ubiquitinated by E3 ligases. Proteins, such as epsin, Eps15, and Eps15R, contain ubiquitin-interacting motifs (UIMs, a type of ubiquitin-binding domain [UBD]), and bind to ubiquitinated RTKs. UIM proteins bind to the appendage domains (mainly α) of AP-2, and epsin can also directly bind to the terminal domain of clathrin heavy chain (main components of clathrin triskelions). Interaction of RTKs with UIMs can occur before the recruitment of receptors into CCP and/or with UIMs present in assembled CCPs. The heterotetrameric AP-2 complex consists of four subunits: α , β 2, μ 2, and σ 2. The trunk/core domain of AP-2 is formed by μ 2, σ 2, and the core domains of α and β 2. Yxx Φ and LL motifs directly interact with the μ 2 and σ 2/ β 2 subunits of AP-2, respectively. NPxY motifs interact with phosphotyrosine-binding domain (PTB) of adaptor proteins, such as Disabled-2, ARH, and NUMB, which themselves bind to the clathrin terminal domain and AP-2 appendage domains. The hinge region of β 2 (between trunk and appendage) binds to clathrin heavy chain.

as Cbl. Cbl binds to and ubiquitinates EGFR, FGFR, PDGFR, VEGFR, CSF-1R, hepatocyte growth factor (c-Met), macrophage stimulating protein (Ron) and ephrin (EphR) receptors, as well as other RTKs (Levkowitz et al. 1999; Miyake et al. 1999; Wilhelmsen et al. 2002; Duval et al. 2003; Penengo et al. 2003; Marmor and Yarden 2004; Fasen et al. 2008). c-Cbl knockout, siRNA depletion of c-Cbl and Cbl-b, or overexpression of inactive Cbl mutants inhibit internalization of CSF-1R, EGFR, c-Kit, and several other RTKs (Lee et al. 1999; Levkowitz et al. 1999; Zeng et al. 2005; Huang et al. 2006). These studies implicate Cbl as a key mediator of RTK endocytosis. Besides Cbl, another

RING family E3, Nrdp1, ubiquitinates an EGFR homolog ErbB3 in a ligand (neuregulin)-independent manner, but this ubiquitination is augmented by neuregulin stimulation through the increased stability of Nrdp1 (Cao et al. 2007).

Cbl is a multidomain protein capable of interacting with various regulatory proteins. Activity of Cbl and its turnover are regulated by a range of mechanisms involving interaction with Sprouty2 and p85Cool-1, ubiquitination by NEDD4-1, and phosphorylation by Src (Wong et al. 2002; Bao et al. 2003; Wu et al. 2003b; Marmor and Yarden 2004; Thien and Langdon 2005). Therefore, ubiquitination of RTK can be indirectly regulated by these proteins. All three



mammalian isoforms of Cbl (Cbl-b, Cbl-3, and c-Cbl) bind directly to specific phosphorylated tyrosines in RTKs, and long Cbl species (c-Cbl and Cbl-b) bind EGFR, c-Met, PDGFR- β , stem cell factor receptor (c-kit), and other RTKs indirectly through the SH2 adaptor protein Grb2 (Batzer et al. 1994; Peschard et al. 2001; Waterman et al. 2002; Jiang and Sorkin 2003; Li et al. 2007; Sun et al. 2007). The key role of Grb2 in ubiquitination and internalization of RTKs via Cbl has been shown using multiple experimental approaches in mammalian cells (Peschard 2001; Jiang 2003; Huang and Sorkin 2005; Johannessen 2006; Li 2007). In *Drosophila* and *Caenorhabditis elegans*, D-Cbl L binds to Grb2/Drk and Sli-1/Cbl binds to Grb2/SEM-5 to promote EGFR endocytosis (Yoon et al. 2000; Wang and Pai 2011). Finally, Grb2 and Cbl are colocalized with and remain bound to activated EGFR in CCPs (de Melker et al. 2001; Jiang et al. 2003; Johannessen et al. 2006). Collectively, these studies suggest that Grb2-Cbl complexes are required for the recruitment of RTKs into CCPs and, thereby, play an important role in receptor internalization.

The discrepancy between the requirement of Grb2 and Cbl for EGFR internalization and the lack of the role of EGFR ubiquitination sites might be explained by the existence of an unidentified receptor-associated protein that is (1) ubiquitinated by Cbl, and (2) mediates the internalization of the receptor (Fig. 3). It is important to note that the role of Grb2 and Cbl may be unrelated to ubiquitination. For instance, the SH2 domain of Grb2 binds to Tom1L protein that interacts with clathrin, thus linking EGFR to coated pits (Liu et al. 2009). It is unclear, however, how the SH2 domain of Grb2 binds simultaneously to both EGFR and Tom1L. Leucine repeat kinase Lrrk1 also binds to EGFR through Grb2 and promotes EGFR endocytosis (Hanafusa et al. 2011). On PDGF stimulation, Grb2 binds to PDGFR- β and the SH3 domain of Grb2 interacts with dynamin to facilitate internalization of the receptor (Kawada et al. 2009). A Cbl-interacting adaptor protein, CIN85, interacts with a plethora of partners through its five SH3 domains and is implicated in internalization of various RTKs

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(Petrelli et al. 2002; Soubeyran et al. 2002; Szymkiewicz et al. 2002; Kowanetz et al. 2003; Schmidt et al. 2004; Buchse et al. 2011). However, the effects of knockdown or knockout of CIN85 on clathrin-mediated RTK internalization and the localization of CIN85 in CCPs have not been shown. It can be concluded that although the key role of Cbl and Grb2 in RTK endocytosis is well established, the role of RTK ubiquitination in this process remains unclear, and ubiquitination-independent mechanisms involving Grb2 and Cbl are not fully supported by functional and localization data.

Role of NEDD4 Family and Other E3 Ligases

The second large family of E3s, the HECT domain-containing NEDD4 ligases, directly interact with substrate PPxY motifs through their WW domains (Fig. 2) (Rotin and Kumar 2009). NEDD4-2 was shown to ubiquitinate TrkA receptor by binding to its carboxyl terminus (Arevalo et al. 2006). Another NEDD4 family member, AIP4/Itch, binds to the PPxY motif in ErbB4 (Sundvall et al. 2008). NEDD4-1 binds to an unconventional sequence of FGFR1 and ubiquitinates this receptor (Persaud et al. 2011). siRNA depletion of NEDD4 family or overexpression of their catalytically inactive forms inhibited endocytosis of TrkA, ErbB4, and FGFR1 (Arevalo et al. 2006; Sundvall et al. 2008; Persaud et al. 2011). IGF-1R and IR ubiquitination involves an indirect binding of NEDD4-1 through the Grb10 adaptor (Fig. 2) (Vecchione et al. 2003; Monami et al. 2008). The SH2 domain of Grb10 is capable of simultaneous binding to the C2 domain of NEDD4-1 and IGF-1R (Huang and Szebenyi 2010). Expression of Grb10 and NEDD4-1 mutants significantly reduced endocytosis of IGF-1R (Vecchione et al. 2003; Monami et al. 2008). Genetic knockout of NEDD4-1 caused reduction of IGF-1R on the cell surface of mouse fibroblasts, suggesting that the role of NEDD4-1 in this system is more complex (Cao et al. 2008). The latter study proposed that NEDD4-1 regulates Grb10 levels. In the absence of NEDD4-1, Grb10 is up-regulated, which results in increased endocytosis of IGF-1R. Although NEDD4-1/Grb10 complex

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appears to be involved in endocytosis of IR/IGF-1Rs, the localization of these proteins during early internalization events has not been determined.

The mechanisms by which IGF-1R is regulated by ubiquitination are convoluted because IGF-1R can also be ubiquitinated by the RING domain E3 ligase Mdm2, which is itself associated with IGF-1R through interaction with β -arrestins, and by c-Cbl (Girnita et al. 2005; Sehat et al. 2008). Indeed, siRNA knockdown of individual E3s implicated in IGF-1R ubiquitination did not affect IGF-1R endocytosis (Mao et al. 2011). The IGF-1R example of the potentially redundant role of multiple E3s in ubiquitination of a single RTK can be extended to EGFR (Cbl, Smurf2) (Levkowitz et al. 1999; Ray et al. 2011), ErbB4 (Itch, NEDD4, and WW1) (Sundvall et al. 2008; Li et al. 2009; Zeng et al. 2009), and TrkA (Cbl, NEDD4-2, and TRAF-6/UbcH7) (Geetha et al. 2005; Arevalo et al. 2006; Takahashi et al. 2011). Ubiquitination of a single RTK by different E3 ligases may result in different functional outcomes (Mao et al. 2011; Ray et al. 2011). Likely the contribution of different E3s is cell-type specific, depends on the experimental conditions, and can be indirect in some cases.

UBIQUITIN-BINDING PROTEINS IN CCPs

The model assuming that ubiquitin moieties target RTKs to CCPs implies that three UBD-containing proteins residing in CCPs (epsin-1/2, Eps15, and Eps15R) are involved in RTK internalization (Fig. 3). Binding of these proteins to ubiquitin moieties *in vitro* is well documented (Hawryluk et al. 2006), and coimmunoprecipitation of epsin-1 and Eps15 with activated EGFR has also been shown (Sigismund et al. 2005). There are, however, contrasting data regarding the importance of these proteins in EGFR internalization. siRNA knockdown of epsin-1 completely abolished EGF internalization in one study (Kazazic et al. 2009), whereas others report that individual or simultaneous depletion of epsin-1, Eps15, and Eps15R did not specifically block the CME of EGFR (Huang et al. 2004; Sigismund et al. 2005; Chen et al.

2009). Because epsin and Eps15/R contain multiple protein–protein interaction modules, overexpression of these proteins is not informative to their function (Sugiyama et al. 2005). Furthermore, epsin and Eps15/R may have distinct functions in cargo recruitment. Epsin is distributed throughout the entire CCP, whereas Eps15 is located at the edge of pits (Tebar et al. 1996; Kazazic et al. 2009). In general, direct interactions of epsins and Eps15/R with ubiquitinated RTKs are difficult to show, and the precise role of these three UBD-containing proteins in redundant or sequential recognition of ubiquitinated RTKs remains to be elucidated.

OTHER MECHANISMS OF RTK INTERNALIZATION

A tumor suppressor protein, RALT/Mig-6, was shown to promote EGFR endocytosis by a receptor-kinase-independent mechanism (Frosi et al. 2010). RALT/Mig6 binds to the EGFR kinase and inhibits its activity. RALT/Mig-6 is also capable of binding to AP-2 and intersectin, thus mediating internalization of EGFR through CCPs. Acetylation of three lysines at the carboxyl terminus of EGFR may also support internalization (Fig. 2). Mutation of these residues together with ubiquitination sites in the kinase domain of EGFR resulted in inefficient recruitment of EGFR into CCP, inability to phosphorylate the β 2 subunit of AP-2, and significant inhibition of the EGFR CME (Goh et al. 2010). The precise role of EGFR acetylation in internalization is unknown, but could be related to the regulation of the receptor-kinase-substrate interactions. A unique Eph receptor system involves activation of Eph endocytosis by a membrane-anchored ligand (ephrin) (Pitulescu and Adams 2010). In neurons, endocytosis of ephrin-EphA4 receptor complex is mediated by the Rho family guanine exchange factor (GEF) Vav2 through an actin-dependent process (Cowan et al. 2005). Additionally, RIN1 (Ras binding and Rab5 GEF) is implicated in EphA4 endocytosis (Deininger et al. 2008). It is unclear, however, whether EphA4 endocytosis mediated by Vav2 or RIN1 is clathrin dependent.



POSTENDOCYTIC SORTING OF RTKs: MORPHOLOGY AND PATHWAYS

Endocytic carriers formed by CME and CIE deliver cargo to EEs located in the cell periphery. EEs have vesicular and tubular subcompartments; they can fuse to each other while moving along microtubules toward the centriole/Golgi area of the cell (Fig. 1). In the specialized and extreme example of neuronal cells, ligand-bound Trk receptors are internalized in the axonal terminal and transported for a long distance in EEs in a microtubule-dependent manner to the neuronal soma. The mechanisms and functions of the retrograde transport of endocytosed neurotrophin receptors were recently reviewed (Wu et al. 2009).

Movement toward the pericentriolar region is accompanied by the endosome maturation process that involves enlargement of the vesicular subcompartment, reduction in the amount of tubular extensions, and appearance of ILVs that are characteristic of MVBs. Changes in the biochemical composition of endosomes and an increasing luminal acidification also occur. Sorting of RTKs takes place in MVBs (Figs. 1 and 4). RTKs that are located in the limiting membrane of EEs and MVBs are capable of recycling back to the cell surface by moving to tubular extensions of these endosomes. RTKs that are packaged into ILVs are incapable of recycling and remain in MVBs that mature into late endosomes. Proteases and other lysosomal enzymes are delivered to MVB/late endosomes from the Golgi complex, leading to degradation of ILVs and RTKs.

MOLECULAR MECHANISMS OF RTK ENDOSOMAL SORTING

The current model of postendocytic sorting suggests that ubiquitinated RTK is recognized on the limiting membrane of a MVB by the heterodimeric complex of Hrs (hepatocyte growth factor regulated tyrosine-kinase substrate) and STAM1/2 (signal transducing adaptor molecule 1 and 2), which is the main component of ESCRT (endosomal sorting complexes required for transport)-0 (Fig. 4). UBDs in the Hrs and

STAMs bind ubiquitinated RTKs and, possibly with the help of clathrin lattice associated with Hrs, retain RTKs from free diffusion in the limiting membrane and tubular extensions of MVBs, thus preventing RTK recycling. It has also been proposed that ubiquitinated EGFR interacts with GGA3, another UBD-containing adaptor, in EE before interacting with ESCRT-0 (Puertollano and Bonifacio 2004). Further, ESCRT-0 facilitates sequential recruitment of ESCRT-I, ESCRT-II, and ESCRT-III onto the MVB membrane. It is hypothesized that ubiquitinated cargo is passed along to ESCRT-I and -II, and ultimately packaged into ILV via a process of inward membrane invagination mediated by ESCRT-III (Henne et al. 2011).

The MVB sorting model is well supported by multiple lines of evidence. Degradation of EGFR, FGFR-2, and IGF-1R was highly sensitive to mutations of ubiquitin-conjugation and Cbl binding sites (Huang et al. 2006; Haugsten et al. 2008; Mao et al. 2011). Ubiquitin-deficient EGFR mutant was poorly incorporated into ILVs (Eden et al. 2011). The experimental manipulations with E3 ligases, their adaptors (Grb2 and Grb10), and other regulatory proteins cited above in the internalization section also have strong effects on the ligand-induced degradation of RTKs. For example, modulators of Cbl-mediated EGFR ubiquitination, such as Sprouty2, low-density lipoprotein receptor-related protein, Lrig1, and CIN85, also modulate the rate of lysosomal degradation of EGFR (Wong et al. 2002; Gur et al. 2004; Takayama et al. 2005; Dikic and Schmidt 2007; Ronning et al. 2011). Furthermore, the effects of overexpression of dominant-negative mutants of ESCRT components and their down-regulation by knockouts, knockdowns, and a microRNA on the lysosomal degradation of RTKs have been shown (Bache et al. 2003; Jekely and Rorth 2003; Bowers et al. 2006; Ewan et al. 2006; Wurdinger et al. 2008; Belleudi et al. 2009).

It should be emphasized that according to the ESCRT sorting model, RTK ubiquitination, and therefore, ligand binding, kinase activity, and Cbl association must be maintained until the formation of an ILV. To avoid the loss of ubiquitin, the cargo must also be deubiquiti-

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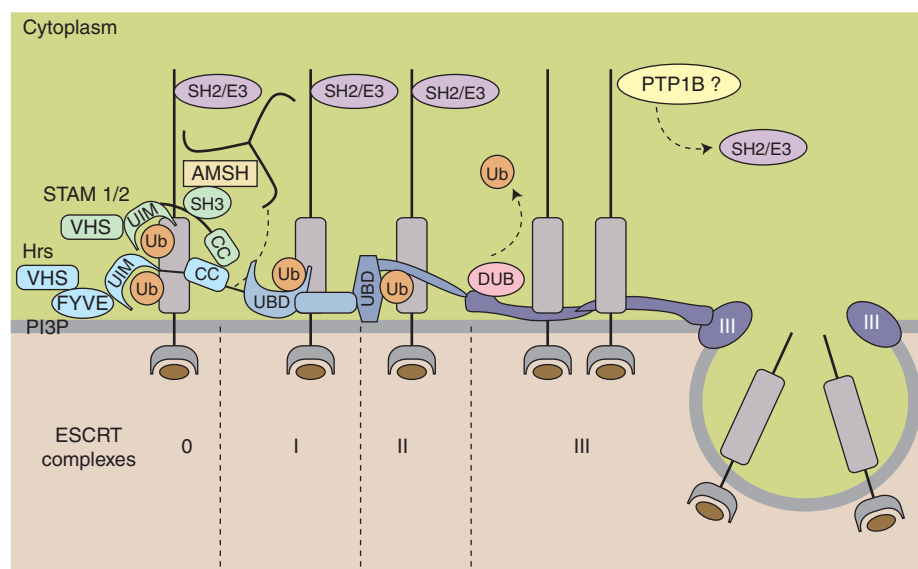


Figure 4. Hypothetic mechanism of RTK sorting into intraluminal vesicles of MVBs. Ubiquitinated RTK is recognized by UIMs of Hrs and STAM1/2 (ESCRT-0). Hrs is anchored to endosomal membrane through the interaction of its FYVE domain with phosphatidylinositol 3-phosphate (PI3P). Hrs also contains a coiled-coil domain that interacts with a similar domain of STAM, VHS domain, and a clathrin-binding motif. Binding of clathrin triskelions to Hrs nucleates an assembly of a flat clathrin lattice that further recruits additional Hrs molecules, leading to trapping of ubiquitinated cargo in the “Hrs microdomain.” STAM contains the SH3 domain known to interact with AMSH. Accumulation of Hrs onto the endosomal membrane facilitates translocation of TSG101 and other components of ESCRT-I, and sequentially, components of ESCRT-II from cytosol to the MVB membrane. TSG101 and the ESCRT-II component EAP45/Vps36 have UBDs that may interact with ubiquitinated RTK. Ubiquitinated receptors appear to be transferred from ESCRT-0 to ESCRT-I and -II owing to increasing local concentrations of the latter complexes. ESCRT-III does not have ubiquitin-binding domains, and presumably traps receptors into forming ILVs by assembling into concentric hetero-oligomeric filaments and restricting diffusion of receptors. After formation of an ILV, ESCRT-III is disassembled by the Vps4 complex. The ESCRT model is reviewed by Teis et al. (2009). Before RTK entering the ILV, DUBs are proposed to remove ubiquitin from the receptor. Furthermore, receptor-associated proteins, such as SH2 adaptors and ubiquitin ligases, must also be removed before sequestration of receptors into ILV, possibly by means of receptor dephosphorylation by phosphotyrosine phosphatases like PTP1B (Eden et al. 2010). In addition, RTKs may facilitate ILV formation by phosphorylating annexin 1 (not shown) (White et al. 2006).

nated before entrance into ILVs. The requirement of such a tightly timed RTK deubiquitination for ILV incorporation, however, has not been directly shown. The deubiquitination enzyme (DUB) AMSH (associated molecule of SH3 domain of STAM) has been shown to associate with ESCRT-0 and also with the ESCRT-III component CHMP3 (Tanaka et al. 1999; Ma et al. 2007). AMSH directly deubiquitinates EGFR and PDGFR, and therefore decreases their degradation (McCullough et al. 2004, 2006; Bowers et al. 2006). Another DUB involved in cargo sorting is Usp8, which affects

EGFR degradation by controlling the stability of STAM (Urbe et al. 2006) and Eps15 (Mizuno et al. 2006). However, there are contrasting effects of Usp8 knockdown on EGFR and c-Met degradation (Mizuno et al. 2005; Row et al. 2006). A conditional mouse knockout of Usp8 leads to decreased levels of EGFR, c-Met, and ErbB3 (Niendorf et al. 2007). Although it is clear that AMSH, Usp8, and possibly other DUBs play an important role in RTK sorting, further investigation is needed to distinguish between their function in deubiquitination of RTKs before (“sorting-negative” effect) or after



ubiquitin-dependent RTK-ESCRT interactions (“sorting-positive” effect) and in deubiquitination of ESCRT proteins themselves.

The importance of a specific type of ubiquitination during RTK sorting in MVBs is another unresolved issue. Proteins can be modified by attachment of a single ubiquitin to a single lysine residue (monoubiquitination) or several lysines (multimonoubiquitination). Lysines in the ubiquitin itself can further serve as substrates for ubiquitin conjugation resulting in the formation of polyubiquitin chains. Mass spectrometry studies revealed that EGFR is ubiquitinated by Lys63-linked chains (Huang et al. 2006). Similarly, TrkA was proposed to be either monoubiquitinated or Lys63-chain polyubiquitinated (Geetha et al. 2005; Arevalo et al. 2006). In contrast, IGF-1R was found to be Lys48- and Lys29-polyubiquitinated during antibody-driven down-regulation (Mao et al. 2011). Unlike Lys48-linked chains that target proteins to proteasome, Lys63-linked polyubiquitination and monoubiquitination have nonproteosomal functions (Adhikari and Chen 2009). Importantly, Lys63-linked ubiquitin chains and monoubiquitin are recognized by UBDs in a similar manner (Varadan et al. 2004). Therefore, multimonoubiquitination and Lys63-polyubiquitination provide a platform for simultaneous RTK interactions with multiple UBDs in ESCRTs and, therefore, increase the avidity of weak ubiquitin-UBD interactions and the efficiency of sorting into ILVs (Ren and Hurley 2010). Considering the ample amount of evidence supporting the role of Lys63 chains in degradation of various ubiquitinated cargo, it can be proposed that Lys63-linked polyubiquitination is the major molecular signal involved in the endocytic sorting of mammalian RTKs. However, this hypothesis and its applicability to different subtypes of RTKs require further experimentation.

Finally, it should be noted that other mechanisms that do not appear to be related to ubiquitination have been implicated in RTK endosomal sorting. The LL motif in the carboxyl terminus of EGFR is important for EGFR degradation (Huang et al. 2003). Binding of RALT/MIG-6 associated with EGFR to the endosomal

SNARE Syntaxin 8 also promotes EGFR degradation (Ying et al. 2010). The precise mechanisms underlying these regulations are poorly understood, and whether ESCRTs are involved is not determined.

RTK RECYCLING

As internalized activated RTKs are efficiently targeted for lysosomal degradation, it is generally assumed that recycling of these receptors is minimal. This notion, together with technical difficulties in recycling rate measurements, translates into a scarcity of information regarding RTK recycling mechanisms. Considerable recycling of ligand-receptor complexes has been shown for IR (Huecksteadt et al. 1986), EGFR (Sorkin et al. 1991), and TrkA (Zapf-Colby and Olefsky 1998). EGF-EGFR complexes are rapidly recycled back to the cell surface from peripheral EE and with a slower kinetics from pericentriolar endosomes (Fig. 1) (Sorkin et al. 1991). The fast recycling pathway or “retroendocytosis” is mediated by constitutive formation of recycling carriers from peripheral EE, whereas a slower pathway involves a sorting step in MVBs and late recycling compartments. Studies of the kinetics of EGF-EGFR sorting showed that the degradation pathway is saturable, and therefore, the size of the recycling pool of EGF-EGFR complexes is proportional to the concentration of internalized EGF-EGFR complexes (French et al. 1994). For instance, in cells expressing high levels of EGFR and stimulated with high EGF concentrations, up to 80% of internalized EGF-receptor complexes were recycled (Sorkin et al. 1991). In addition, unoccupied EGFRs that release EGF in endosomes were recycled back to the plasma membrane (Masui et al. 1993). It is estimated that ligand-occupied receptors recycle with a slower rate than unoccupied EGFR (Resat et al. 2003). Furthermore, a pool of unoccupied, and therefore, recycled EGFR in endosomes is significantly larger when EGFR is activated by ligands like transforming growth factor α that displays a higher pH sensitivity of binding and dissociates from the receptor in endosomes, as compared with EGF that remains mostly receptor bound

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(French et al. 1995; Roepstorff et al. 2009). Also, alterations that are inhibitory to the kinase activity and ubiquitination, such as threonine phosphorylation in the juxtamembrane domain of EGFR by protein kinase C (PKC) or mutations of ubiquitination sites, elevate recycling of EGFR (Bao et al. 2000; Eden et al. 2011). Likewise, activation of PKC was necessary for recycling of PDGFR- β (Hellberg et al. 2009).

The kinetic analysis of EGFR trafficking is in line with the model whereby recycling is the default pathway of the cargo that is not trapped by ESCRT-0 in MVBs. Therefore, RTKs recycle through the pathways and compartments common with nutrient carrier receptors like the transferrin receptor. These recycling pathways are regulated by Rab4 in peripheral EE and Rab11 (and homologous Rabs, such as Rab22) in pericentriolar endosomes (Grant and Donaldson 2009). Specific “recycling” sequence motifs described for other classes of receptors have not been found in RTKs (Hanyaloglu and von Zastrow 2007). Recently, however, GGA3 was shown to bind c-Met via the Crk adaptor and promote c-Met recycling, suggesting that specific “recycling targeting” mechanisms may exist (Parachoniak et al. 2011).

CONCLUDING REMARKS

An emerging common theme in the RTK endocytosis literature is that RTK internalization is a highly robust process resistant to singular perturbations (e.g., mutations or altered expression of components of the endocytosis machinery). Possible explanations behind the robustness of the endocytic process relate to the existence of clathrin-dependent and -independent mechanisms. Furthermore, CME can itself be regulated by several redundant mechanisms. In the example of EGFR, simultaneous mutation of AP-2 binding motifs, multiple ubiquitin-conjugation and acetylation sites in EGFR is necessary to substantially suppress this receptor internalization via the CME pathway (Goh et al. 2010). Similarly, participation of AP-2 binding motifs and ubiquitination has been implicated in internalization of other RTKs. Moreover, ubiquitination of a single RTK can be mediated by

several E3 ligases. In contrast, lysosomal targeting is mediated by a single major mechanism—ubiquitination—and, therefore, this step of trafficking is less robust and highly sensitive to the regulation of the ubiquitination and deubiquitination systems. In light of the redundancy of endocytosis mechanisms in mammalian cell culture in vitro, it is important to gear future research toward studying the mammalian RTK endocytosis in vivo so that the mechanistic analysis of endocytic pathways that are physiological and highly relevant to human disease can be revealed.

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