Breakdown of endocytosis in the oncogenic activation of receptor tyrosine kinases

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Abella JV, Park M. Breakdown of endocytosis in the oncogenic activation of receptor tyrosine kinases. Am J Physiol Endocrinol Metab 296: E973–E984, 2009.—There is increasing evidence to support the concept that the malignant behavior of many tumors is sustained by the deregulated activation of growth factor receptors. Activation of receptor tyrosine kinases (RTKs) by their respective ligand(s) initiates cellular signals that tightly modulate cell proliferation, survival, differentiation and migration to ensure normal tissue patterning. Therefore, uncontrolled activation of such signals can have deleterious effects, leading to oncogenesis. To date, deregulation of most RTKs has been implicated in the development of cancer, although the mechanisms that lead to their deregulation are not yet fully understood (10). RTK endocytosis, the internalization and trafficking of receptors inside the cell, has long been established as a mechanism to attenuate RTK signaling. However, RTKs have been demonstrated to continue to signal along the endocytic pathway, which contributes to the spatio-temporal regulation of signal transduction. This review will focus on recent advances linking defective endocytosis of RTKs in the development of cancer.

Cbl; ubiquitination; receptor downregulation

RECEPTOR TYROSINE KINASES (RTKs) are single-pass transmembrane proteins whose intrinsic catalytic activity, to phosphorylate tyrosine residues, is regulated through ligand binding to the extracellular domain of the protein. Engagement with ligand promotes receptor dimerization or oligomerization, inducing conformational changes within the catalytic domain that allow binding of ATP and activation of the enzyme. Kinase activation first promotes phosphorylation of tyrosine residues within the activation loop of the catalytic domain, which helps to stabilize the activation state (66). The subsequent transphosphorylation of specific tyrosine residues located outside of the catalytic domain generates phosphotyrosine-dependent docking sites for proteins that contain src homology 2 (SH2) or phosphotyrosine-binding domains. These domains recognize phosphotyrosine residues in the context of their surrounding amino acids (125), promoting the formation of a ligand-dependent signaling complex. Proteins recruited to RTKs include those with enzymatic activity, such as phospholipase Cγ, phosphatidylinositol 3′-kinase (PI3K), and cytoplasmic tyrosine kinases of the Src superfamily. In addition, adaptor and scaffold proteins that lack enzymatic activity act to assemble and recruit networks of signaling proteins to the RTK through the presence of additional protein-protein interaction domains (124). These signaling complexes relay and amplify signals that ultimately lead to changes in gene transcription as well as nontranscriptional changes that promote remodeling of the actin cytoskeleton to alter cell shape, motility, and adhesion (124).

Multiple mechanisms lead to the oncogenic activation of RTKs. These include receptor amplification or transcriptional overexpression, chromosomal translocation, point mutation, and the formation of an autocrine loop (85). Of the 58 genes encoding RTKs (10), somatic mutations have now been identified in each of these in human cancer (47). Many other studies have also begun to catalog the expression of RTKs, correlating changes in expression with human malignancies (111, 173). However, it still remains to be tested whether these mutations or alterations in expression are selected for and contribute to tumor formation or are simply passenger mutations. Genomic amplification, overexpression, or mechanisms that inhibit receptor degradation can result in an increased concentration of RTK levels at the cell surface. This in turn can lead to enhanced dimerization and activation of the receptor in the absence of ligand as well as increased sensitivity to low levels of ligand. Members of the ErbB/epidermal growth factor receptor (EGFR) family are commonly overexpressed in human tumors, where ErbB2/HER2 is amplified in ≈20% of human breast cancers (114) and the ErbB1/EGFR in 80% of head and neck tumors, 40–50% of glioblastomas, and <20% of various squamous cell carcinomas (46, 187, 192). Chromosomal translocations, which are prevalent in hematological malignancies, but also occur in solid tumors, generally fuse a protein dimerization motif to the cytosolic kinase domain of an RTK (106, 141, 161). This removes the extracellular ligand-binding domain of the RTK but results in a constitutively active receptor, mediated through protein dimerization in the absence of ligand.
Regulated event and not merely the consequence of receptor degradation (150). However, whether PTPs directly regulate endocytosis of RTKs still remains to be determined.

Downregulation of RTKs Through the Endocytic Pathway

The endocytic pathway was established to act as the major mechanism for downregulation of the EGFR (182). Although several distinct pathways for RTK internalization have been identified (107), these are as yet not extensively studied for RTKs, and for the purposes of this review, we shall focus solely on the clathrin-dependent pathway. Following ligand stimulation, internalized receptors are subject to two distinct fates: 1) recycling back to the plasma membrane or 2) degradation via the lysosomal pathway (Fig. 1). The first identified and best-studied route for entry of RTKs into the cell is the clathrin-dependent pathway. Briefly, receptor-ligand complexes are recruited by adaptor molecules such as adaptor protein-2 (AP-2), epidermal growth factor receptor pathway substrate 15 (Eps15), and CIN85 (Cbl-interacting protein, 85 kDa) into clathrin-coated pits (CCPs) within the plasma membrane (Fig. 1). These pits eventually bud into the cell to form clathrin-coated vesicles (CCVs) through a scission event catalyzed by the enzyme dynamin. CCVs shed their clathrin coat and deliver receptors to the endosomal network. At this point, receptors may become uncoupled from their ligand due to a decrease in pH and recycle back to the plasma membrane or progress down the endocytic pathway to be sorted for lysosomal degradation (42). The rapid removal of RTKs from the cell surface and the subsequent targeting to lysosomal compartments is important to prevent sustained activation from both the plasma membrane (182) and on endocytic vesicles (23), which can lead to cell transformation (103) and tumorigenesis (129).

Ubiquitin

Historically, protein ubiquitination has been demonstrated to target proteins for degradation by the 26S proteasome (37). It is now clear, however, that ubiquitin plays a pivotal role in the endocytic pathway to target RTKs for lysosomal degradation. Protein ubiquitination can have several functions based on the type of ubiquitin chain attached (185). Ubiquitin chains are formed through the subsequent addition of ubiquitin moieties onto specific lysine residues on ubiquitin. Ubiquitin chains formed through the addition to lysine 63 (K63) adopt an extended linear conformation that has been demonstrated to have nonproteolytic functions and act as a protein scaffold (27, 176). Lysine 48 (K48)-linked chains have a very different topology, adopting a closed conformation (29). Whereas K48-linked ubiquitin chains are a signal for proteasomal degradation, K63 chains and mono- or multimonoubiquitination of proteins is not. Monoubiquitin and K63-linked chains act as docking sites for proteins that contain ubiquitin-interacting domains or motifs (185). The discovery of domains able to bind to ubiquitin is expanding rapidly, bringing the current total to 16 (69). It is evident that different domains have different affinities for the type of ubiquitin linkage, and this undoubtedly provides an important level of regulation in the ubiquitin-signaling pathway, which parallels that of protein phosphorylation (185).

Initial studies in yeast demonstrated that receptor ubiquitination is required for internalization of transmembrane proteins...
Subsequently, using the cell surface uracil permease transporter as a model, it was established that, although monoubiquitination was sufficient to induce transporter endocytosis, K63-linked polyubiquitin chains were required for efficient endocytosis and transporter turnover (43). Later studies in mammalian cells, however, proved that receptor ubiquitination is not essential for internalization (1, 26, 62) but may regulate this process (158). Instead, in mammalian cells, receptor ubiquitination is a post-translational modification that can modulate the internalization and degradation of receptors in the endocytic pathway. Ubiquitination marks receptors for proteasomal degradation, which is essential for the turnover of many cellular proteins.

Fig. 1. Receptor tyrosine kinase (RTK) downregulation through the endocytic pathway. Growth factor activation of RTKs results in receptor phosphorylation and recruitment of the E3 ligase Cbl. Cbl mediates ubiquitination of RTKs and perhaps some endocytic adaptor molecules, which gather RTKs into clathrin-coated pits (CCPs; green RTKs). Uncoupling RTKs from Cbl-mediated ubiquitination (red RTKs) can have moderate to no effect on RTK internalization, depending upon the RTK. CCPs bud into the cytosol to form clathrin-coated vesicles (CCVs). CCVs eventually shed their clathrin coat and fuse with early endosomes. RTKs can either be recycled back up to the membrane, particularly if they are not ubiquitinated, or continue down the endocytic pathway. Ubiquitinated RTKs are recognized by components of the endosomal sorting complex required for transport (ESCRT) machinery, which recruit receptors onto a flat, bilayered clathrin lattice on the sorting endosome. RTKs are subsequently sorted for internalization into the endosomal lumen. Nonubiquitinated RTKs are not efficiently recruited for internalization and can remain on the endosomal membrane as active signaling molecules. Endosomes that become filled with intralumenal vesicles containing RTKs, known as multivesicular bodies (MVBs), fuse with lysosomes to degrade the lumenal contents. Chimeric RTKs, which result from chromosomal translocations, are no longer targeted to the plasma membrane, precluding them from downregulation through the endocytic pathway. If these chimeric fusion proteins are ubiquitinated (mono or K63), they are not recognized by components of the 26S proteasome for degradation. Cbl may be sequestered from mediating RTK ubiquitination by proteins such as Src, Sprouty2, cortactin, E5, and β-PiX. Proteins of the endocytic pathway written in orange have been identified in human cancers as being mutated and over- or underexpressed or are frequent substrates for chromosomal translocations. Endophilin II is recruited to the plasma membrane by CIN85 (Cbl-interacting protein, 85 kDa) and induces negative membrane curvature to produce CCPs (130, 163). Huntington interacting protein 1 (HIP1), clathrin assembly myeloid lymphoid leukemia (CALM/Ap180), and Numb are clathrin coat adaptor molecules. Rabaptin5 is a Rab5 GAP involved in regulating endosome fusion (164) and has been found as a chromosomal translocation product with the platelet-derived growth factor receptor-β (PDGFRβ) (100). AP-2, adaptor protein 2; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; STAM, signal-transducing adaptor molecule; Eps15, epidermal growth factor receptor pathway substrate 15. Eps15b is the isoform that functions in ESCRT 0 (145), hepatocellular carcinoma-related protein 1 (HCRP1), and tumor susceptibility gene 101 (TSG101).
cells, monoubiquitination or K63 polyubiquitination of RTKs is required to target them for efficient lysosomal degradation (7, 12, 49, 63, 105, 110, 177). Both monoubiquitin and K63-linked ubiquitin chains act as sorting signals that are recognized by proteins of the endosomal sorting complex required for transport (ESCRT) complex, which contain ubiquitin-interacting motifs (UIM) (184, 185). ESCRTs recruit and orchestrate receptors for internalization into the endosomal lumen, creating a multivesicular body (MVB) (Fig. 1) (17). For example, hepatocyte growth factor-regulated tyrosine kinase substrate and signal-transducing adaptor molecule are part of the ESCRT 0 complex, and both contain UIMs (59, 174). These recognize ubiquitinated RTKs and recruit them onto a specific domain on the endosomal membrane, defined by a flat bilayered clathrin lattice (147) (Fig. 1). Once recruited onto this limiting membrane, ubiquitinated receptors are processed by ESCRT complexes I–III, which contain several ubiquitin-binding proteins, including tumor susceptibility gene 101 (TSG101) as well as others (167). ESCRT complexes can recruit deubiquitinating enzymes, which may be required to remove ubiquitin from RTKs prior to their internalization into the endosomal lumen (3, 97, 144). Upon internalization into the MVB, fusion with lysosomes results in RTK degradation through the action of luminal acid-dependent proteases (76, 132). Therefore, disruption of RTK ubiquitination or of the ESCRT complex has the potential to enhance receptor stability, prolong signaling, and induce cell transformation.

Role of Cbl in RTK Downregulation

The E3 ubiquitin ligase Cbl acts as a key negative regulator of RTKs, promoting the ubiquitination of activated receptors to target them for efficient internalization into MVBs and subsequent lysosomal degradation. In mammals, the Cbl family of ubiquitin ligases includes Cbl, Cbl-b, and Cbl-3 (115). All three members are highly homologous in their amino terminus, which consists of a tyrosine kinase-binding (TKB) domain, containing a variant SH2 domain that engages with specific phosphotyrosine residues in RTKs. A RING domain within the TKB and mediates transfer of ubiquitin to the RTK (169). Recruitment of Cbl to RTKs leads to Cbl phosphorylation, which is required for efficient E3 ligase activity (93).

Loss of Function of Cbl Proteins in Human Cancer

Cbl was first identified as a retroviral oncogene (v-Cbl) from the Cas NS-1 retrovirus, which induces pre-β-cell lymphomas and myelogenous leukemia in mice (86). Mutations in Cbl in general act to disable the ubiquitin ligase activity yet allow the recruitment of Cbl to its substrates, such as RTKs. Such mutations uncouple RTKs from Cbl-mediated ubiquitination and efficient lysosomal degradation. Several distinct mutations in Cbl have now been identified in patients with acute myeloid leukemia, acute nonlymphocytic leukemia, and in an acute nonlymphocytic leukemia transgenic mouse model (11, 152, 159). These mutations all cluster around exon 8, resulting in missense mutations in the linker region or deletion of a portion of the RING finger, and result in impaired ubiquitin ligase activity (152). Where studied, expression of these mutant Cbl proteins induces a ligand-independent activation of the Flt3 RTK by inhibiting its ubiquitination and internalization (152).

In addition to mutations within Cbl, several mechanisms that sequester Cbl from RTKs have also been identified in cancer (Fig. 1). The small GTPase Cdc42, involved in actin remodeling, can also inhibit Cbl-mediated EGFR downregulation through sequestration of Cbl via a complex involving β-PIX (190). The adaptor protein Sprouty2 is phosphorylated upon EGF stimulation and binds to the c-Cbl TKB domain (38, 51). This interaction sequesters Cbl from activated EGFRs, decreasing RTK ubiquitination and internalization and promoting sustained EGF-induced MAPK activation (30, 38, 48, 146). Cortactin, which links the actin cytoskeleton to the endocytic machinery, is overexpressed in breast and head and neck carcinomas due to the frequent amplification of the chromosome 11q13 region in these malignancies (123, 156). Overexpression of cortactin in HeLa cells results in a marked inhibition of Cbl-mediated ubiquitination and downregulation of the EGFR, correlating with a reduction in Cbl phosphorilation and recruitment to the EGFR (172). Moreover, depletion of cortactin in head and neck squamous cell carcinoma cell lines accelerated EGF downregulation and attenuated MAPK signaling (172). Members of the EGFR family are frequently activated in cervical cancers induced by human papillomavirus (HPV) type (77). Interestingly, E5, an oncoprotein encoded by HPV16, can form a complex with the EGFR (70) and has been demonstrated to enhance the activation and signaling of the EGFR through several mechanisms, including disruption of the endocytic pathway (165, 171), and more recently by preventing Cbl recruitment and ubiquitination of the EGFR, resulting in delayed downregulation of the receptor (197). Finally, several other proteins have also been shown to sequester Cbl away from mediating RTK downregulation by inducing Cbl degradation. The CD28 receptor promotes T cell receptor signaling by enhancing the downregulation of both c-Cbl and Cbl-b (2, 198). The tyrosine kinases Src and Hck can also interact with c-Cbl, resulting in the ubiquitination and degradation of both proteins (5, 61, 196). Cbl family members can also interact with the HECT E3 ligases Nedd4 and AIP4 (Itch), which result in proteasomal degradation of Cbl (18, 99).

RTK Mutations in Human Cancer Uncouple Cbl-Mediated Ubiquitination

Several RTKs, including the EGF, hepatocyte growth factor/Met, platelet-derived growth factor, colony-stimulating factor-1 (CSF-1), and stem cell factor/c-Kit receptors, recruit the E3 ligase Cbl upon activation and are subsequently ubiquitinated (35, 75, 88, 94, 108, 179, 186, 195). All of these RTKs have been implicated in the development of cancer, and in several cases, oncogenic activation of these RTKs in human cancers can be linked to loss of receptor ubiquitination. The TKB domain of Cbl is recruited to a phosphorytrosine residue of the Met, EGF, and c-Kit receptors (93, 105, 128). Substitution of this tyrosine for a phenylalanine residue uncouples direct Cbl binding to these receptors and inhibits receptor ubiquitination (105, 128, 181).

Met/HGF RTK in Lung Cancer

In the case of the Met receptor, loss of Cbl-mediated ubiquitination results in a receptor that can still internalize but is not efficiently degraded and is now transforming (1, 128). This nonubiquitinated mutant receptor (Y1003F) is no longer tar-
receptor is amplified in with the ubiquitinated wild-type receptor (181). The EGFR site (Y1045F) induces stronger mitogenic signals compared from Cbl-mediated ubiquitination by loss of the Cbl binding
site and are subsequently poorly ubiquitinated (82, 98, 122).

EGFR Family in Glioblastoma

In a similar manner to the Met RTK, uncoupling the EGFR from Cbl-mediated ubiquitination by loss of the Cbl binding site (Y1045F) induces stronger mitogenic signals compared with the ubiquitinated wild-type receptor (181). The EGFR receptor is amplified in ≈40% of glioblastomas, and in many cases the amplified EGFR bears mutations (31, 33, 188). One common alteration involves truncation of the EGFR (EGFRvV), generating a receptor that lacks the Cbl-TKB binding site at Y1045 (41). The most common mutation, deletion of exons 2–7, located in the extracellular domain, generates a truncated EGFR (EGFRvIII) that no longer binds ligand and lacks the dimerization arm of the EGFR (84, 166). However, many studies have demonstrated that this receptor is constitutively phosphorylated, albeit to a weaker degree compared with ligand-stimulated wild-type EGFR (32, 64, 154), and can induce cell transformation in vitro and in vivo (8, 113, 117).

Several reports have shown that the EGFRvIII is uncoupled from Cbl-mediated ubiquitination and/or exhibits low rates of internalization (45, 52, 64, 154). The direct Cbl binding site Y1045 was shown to be hypophosphorylated, thereby compromising Cbl recruitment and receptor ubiquitination (52). However, one study supports that EGFRvIII is appropriately downregulated by the Cbl family of E3 ligases, suggesting that an alternative mechanism of oncogenic activation may be responsible (20). In addition, EGFRvIII was unable to induce phosphorylation of adaptor molecules important for EGFR internalization (e.g., Eps15), resulting in enhanced retention on the plasma membrane, and delayed degradation compared with the wild-type EGFR (45). The low but constitutive autophosphorylation-dependent signaling of the EGFRvIII coupled with inefficient downregulation could explain the transforming ability of this variant receptor. EGFR mutants bearing various deletions in the COOH-terminal tail, where Cbl is recruited, have also been identified in glioblastomas (41), and therefore, these receptors would be predicted to be poorly ubiquitinated and inefficiently downregulated.

EGFR Family in Breast Cancer

Under physiological conditions, Cbl is poorly recruited to other EGFR family members (HER/ErbB2, -3, and -4), which are not targeted for efficient degradation in the lysosome (9, 92, 131). ErbB2, for which there is no known ligand, forms heterodimers with other EGFR family members, the preferred partner being the EGFR. ErbB2 is considered endocytosis impaired; however, the mechanism behind this defect is still under debate. Several studies demonstrate that ErbB2 is retained at the plasma membrane (9, 60, 91, 162), whereas others have reported rapid recycling of ErbB2 (4, 16, 55, 56, 80, 89). Since ErbB2 is amplified in human cancers such as breast and ovary (36, 114), the ability to stimulate downregulation of ErbB2 as a therapeutic strategy has been an active area of research. The discovery that treatment of tumor cells overexpressing HER2 with anti-HER2 antibodies leads to a decrease in cell growth (25) ignited research into this field. Many studies have now shown that monoclonal antibodies can enhance receptor downregulation through the endocytic pathway (21, 153), possibly by promoting an interaction between Cbl and HER2, resulting in receptor ubiquitination and degradation (80). Herceptin (trastuzumab), is an example of a humanized monoclonal antibody against HER2, which is currently being administered with chemotherapy in the treatment of metastatic breast cancer patients (68). The chaperone heat shock protein 90 (Hsp90) interacts with ErbB2, and this association retains the receptor at the plasma membrane (15). Pharmacological inhibitors of Hsp90, such as geldanamycin, have been effective in increasing ErbB2 downregulation by inducing recruitment of the E3 ligase CHIP (COOH-terminus of Hsp70-interacting protein) to ErbB2 and inducing its ubiquitination and cleavage (90, 91, 191, 199). Geldanamycin is currently being evaluated in clinical trials. Importantly, the combined use of trastuzumab with an Hsp90 inhibitor induced recruitment of both Cbl and CHIP E3 ligases, resulting in higher levels of ErbB2 ubiquitination and degradation than with individual treatments (133).

Amplification of ErbB2 drives the formation of EGFR/ ErbB2 heterodimers. This reduces ligand-induced endocytosis and degradation of the EGFR and leads to enhanced EGFR levels at the plasma membrane (53, 112, 180, 189). Mechanically, EGFR/ErbB2 heterodimers lead to a reduction in Cbl recruitment and enhance receptor recycling, thereby decreasing the efficiency of lysosomal degradation of the EGFR (89, 112). Ligand-activated EGFR/ErbB2 heterodimers also show decreased capacity to induce the formation of CCPs, resulting in decreased rates of internalization and prolonged EGFR signaling at the plasma membrane (53). The retention of the complex at the plasma membrane may also prevent receptor dephosphorylation by tyrosine phosphatases present on endomembranes, thereby promoting sustained EGFR activation (120). In contrast, EGFR homodimers, which recruit and become actively ubiquitinated by Cbl, have a greater propensity to continue down the endocytic pathway and are targeted for degradation in the lysosome (89, 189). The ErbB2/ErbB3 partnership in breast cancer may be important for the aggressive nature of cancers with ErbB2 amplification due to the increased retention of the ErbB2/ErbB3 complex at the plasma membrane by ErbB2 coupled with the enhanced mitogenic signaling of ErbB3. Many therapeutic strategies are now based on disrupting heterodimerization of the endocytosis-deficient receptor ErbB2 by using humanized monoclonal antibodies such as pertuzumab (39). These have been effective in rescuing the efficient downregulation of the EGFR and ErbB3 receptors through the endocytic pathway (39, 148).

Kit and CSF-1 RTKs

In a manner similar to the Met and EGF receptors, deletion of a Cbl TKB binding site (Y568) greatly enhances the transforming and mitogenic activity of the stem cell RTK (c-Kit) (57). Many mutations in the juxtamembrane domain of c-Kit identified in human gastrointestinal stromal tumors relieve autoinhibition by the juxtamembrane domain, resulting in ligand-independent activation of the receptor (14). Such mutations would also result in loss of Cbl recruitment and impaired
downregulation, which may contribute to the oncogenicity of these mutant receptors. Loss of the Cbl TKB binding site has also been implicated in the oncogenic deregulation of the CSF-1 receptor (CSF-1R) in myelodysplasia and acute myeloid leukemia (139), where mutation of the Cbl TKB binding site (Y698) enhances the transforming activity of the CSF-1R (143). Notably, the EGFR, c-Kit, and CSF-1R were first identified as the transforming agent of oncogenic retroviruses v-erb-B, v-Kit, and v-Fms, respectively (24, 54, 193). These viral oncogenes lack the tyrosine residues required for recruitment of Cbl (102, 129, 186), indicating that mutations found in RTKs, which uncouple them from Cbl-mediated downregulation, are an evolutionary conserved pathway that is selected for during tumorigenesis.

Chromosomal Translocations: Mistargeting of RTKs from the Endocytic Pathway

Chromosomal translocations resulting in ligand-independent activation of RTKs occur most frequently in hematologic malignancies (106) but also in solid tumors (161). The PDGFR, fibroblast growth factor receptor-1, nerve growth factor receptor, and rearranged during transfection receptor families are frequent targets of chromosomal translocations (106). In general, these events fuse the catalytic domain of RTKs with a dimerization motif derived from another gene. This promotes constitutive dimerization and activation of the kinase in the absence of ligand (141). With the exception of FIG-ROS, which is targeted to the Golgi (13), all RTK-derived fusion proteins, where studied, have lost their amino-terminal signal peptide and transmembrane domain to target them to the plasma membrane. Consequently, these RTK variants are localized to the cytosol. Hence, even if they still recruit Cbl and become ubiquitinated, they fail to enter the endocytic pathway and thus escape downregulation through lysosomal degradation (Fig. 1). For example, one of these chimeric RTK oncoproteins (Tpr-Met), which loses the Cbl TKB binding site following chromosomal translocation, is not reduced in its transforming ability upon the insertion of Cbl-TKB site (101). The oncogenic activity of this chimeric receptor can be attenuated by targeting the receptor to the plasma membrane (101), thereby reconstituting its ability to enter the endocytic pathway, highlighting the importance of RTK endocytosis in receptor downregulation.

Cellular Stress and Receptor Downregulation

Oxidative stress is a common feature of transformed cells and represents an imbalance in redox homeostasis. There is now accumulating evidence that cellular stress signals result in aberrant activation and localization of the EGFR, generating an environment whereby the EGFR no longer undergoes degradation. An increase in free radical concentration can have deleterious effects on cellular functions by damaging DNA (strand breakage), proteins (peptide cleavage), and lipids (lipid peroxidation) (87). Under conditions of high hydrogen peroxide levels, in response to EGF, the EGFR is aberrantly phosphorylated, no longer recruits Cbl, and hence, becomes uncoupled from ubiquitin-mediated degradation (136). In addition, engagement with Eps15, a component of the internalization machinery that is partly dependent on ubiquitin-mediated interactions, is decreased (22). In addition, oxidative stress activates the Src tyrosine kinase, which can promote proteasome-dependent degradation of Cbl (196) and therefore may also contribute to the impaired ubiquitination of the EGFR and endocytic adaptors. Under these conditions, the EGFR remains at the plasma membrane for a prolonged period of time (78) and, once internalized, is retained in a perinuclear compartment, actively signaling (78). Other cellular stresses, such as UV irradiation or inflammatory cytokines, induce serine and threonine phosphorylation of the EGFR on a short segment of its COOH-terminal tail by the p38 stress-induced kinase (121, 178, 200). Under these conditions, the EGFR is not ubiquitinated nor targeted for degradation but is internalized. In response to UV irradiation, the EGFR undergoes sustained serine/threonine phosphorylation and is internalized and retained on early endosomes (121, 200). The inflammatory cytokine tumor necrosis factor-α induces transient serine/threonine phosphorylation of the EGFR, which is rapidly recycled upon receptor internalization (200). Both processes are ubiquitin independent and therefore uncouple the receptor from a degradative pathway. The identification of this alternate mechanism of EGFR-induced internalization has important implications for cancer therapy since chemotherapeutic agents, which have been shown to activate p38 (96), could induce internalization of the EGFR, making tumors refractory to antireceptor antibody therapies, such as Erbitux and Herceptin, that target the extracellular domain of the receptor (200).

Activity of the ubiquitin-activating and -conjugating enzymes, E1 and E2, is also regulated by the redox status of the cell (73, 157). Treatment of tissue with hydrogen peroxide leads to diminished levels of endogenous ubiquitin-protein conjugates (73). This is due to S-thiolation of the active-site sulfhydryl groups during oxidative stress, which prevents the formation of E1 ubiquitin and E2 ubiquitin intermediates (119), the first two steps in the ubiquitination pathway. Any decrease in the cellular capacity to ubiquitinate proteins would disrupt multiple cellular processes, including RTK downregulation.

Disruption of the Endocytic Machinery

In addition to mutations that uncouple RTKs from efficient downregulation through the endocytic pathway, disruption in any of the components of this pathway could effectively delay the internalization, trafficking, and degradation of RTKs. Indeed, there is growing evidence to support a role for aberrant endocytosis in the development of human cancers. For example, the endocytic adaptor Huntington interacting protein 1 (HIP1) is overexpressed in several human cancers, including breast, prostate, and colon tumors, and is associated with poor clinical outcome (135). HIP1 is a clathrin adaptor that promotes clathrin assembly during CCP formation (Fig. 1). Overexpression of HIP1 in NIH3T3 mouse fibroblasts results in enhanced levels of RTKs at the plasma membrane, most likely by interfering with clathrin-mediated internalization (71, 134). Under these conditions EGFR levels remained stable, and EGF stimulation resulted in sustained activation of both MAPK and PI3K pathways, leading to cell transformation. Another endocytic adaptor protein, Numb (149, 151), is involved in regulating the endocytosis of several transmembrane proteins, including the Notch receptor, EGFR, and β-integrins (72, 118, 151, 160). Numb is lost in 50% of human primary breast
carcinomas due to its proteasomal degradation and results in an increase in Notch signaling (126). Since expression of Numb fragments inhibits EGF and transferrin receptor internalization, it is likely that in tumors where Numb is degraded, EGFR signaling will also be upregulated (151). Interestingly, several other endocytic proteins, including Eps15, clathrin assembly myeloid lymphoid leukemia, and endophilin II, have been identified as chimeric fusion proteins as a result of chromosomal translocations in a variety of human leukemias (Fig. 1) (19). Although disruption of endocytosis has not yet been demonstrated to be the mechanism for oncogenic activation, many of these chimeric proteins would no longer be targeted to the correct subcellular location to carry out their normal function. In addition, such chimeric proteins may also act in a dominant negative manner and sequester other important proteins from the endocytic pathway.

Under hypoxic conditions in tumors, many RTKs exhibit prolonged activation through an unknown mechanism, contributing to oncogenesis (40, 83, 127). During hypoxia, endocytosis of the EGFR becomes attenuated due to inefficient early endosome fusion (194). This occurs through the hypoxia-inducible factor-dependent downregulation in transcription of the rabaptin-5 gene (194), an effector of the early endosome Rab5 GTPase (Fig. 1) (164). Importantly, rabaptin-5 RNA levels from human primary clear-cell renal carcinoma and breast tumors with a hypoxic signature were significantly downregulated compared with normal tissue (194).

Several studies in Drosophila have uncovered tumor suppressor roles for components of the ESCRT machinery. Mutant cells for either protein erupted (TSG101, ESCRT I), vps25 (ESCRT II component) or dVps4 (ATPase, ESCRT III), lose cell polarity and contain an accumulation of actively signaling ubiquitinated receptors (Notch and Thickveins) on endosomes (109, 140, 175). Aberrant receptor signaling leads to ectopic secretion of cytokine-like molecules that induce proliferation of surrounding wild-type cells. Importantly, when apoptosis of mutant cells is inhibited, these cells begin to overproliferate, reminiscent of precancerous cells that require a pharmacological intervention.

Conclusions and Perspectives

Deregulation of RTK endocytosis is clearly emerging as a mechanism of oncogenic activation that is selected for in human cancers. Much work has now demonstrated that deviation of RTKs from clathrin-mediated endocytosis can lead to cell transformation. However, the number of identified internalization pathways available to RTKs has multiplied over the past decade (107). As yet, we do not have a clear understanding of what signals regulate which pathway is taken and how each pathway impacts on RTK signaling and stability. Furthermore, we know very little of the contribution of each pathway in cancer progression. Therefore, it is paramount that we develop a better understanding of how RTKs traffic normally and how these processes are disrupted in cancer. This knowledge will benefit us greatly in developing new therapeutic strategies and identifying new potential targets.

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