Escape from Cbl-mediated downregulation: A recurrent theme for oncogenic deregulation of receptor tyrosine kinases

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Deregulation of growth factor receptor tyrosine kinases (RTKs) is linked to a large number of malignancies. This occurs through a variety of mechanisms that result in enhanced activity of the receptor. Considerable evidence now supports the idea that loss of negative regulation plays an important role in receptor deregulation. RTKs are removed from the cell surface via endocytosis and many are subsequently degraded in the lysosome. Lysosomal targeting has recently been linked with receptor ubiquitination. We review here molecular alterations that uncouple RTKs from ubiquitination and implicate loss of ubiquitination as a process that plays a significant role in the pathogenesis of cancer.

Degradation of cellular regulatory proteins following their ubiquitination plays a critical role in controlling multiple physiological processes. Substrates of this pathway include tumor suppressor proteins (p53), cell cycle proteins ($p27^{Kip1}$), and transcription factors (E2F-1, fos, jun, myc, NF-κB). More recently, receptor tyrosine kinases (RTKs), including the colony-stimulating factor-1 receptor (CSF-1R), the epidermal growth factor receptor (EGFR), the hepatocyte growth factor receptor (HGFR/Met), and the platelet-derived growth factor receptor (PDGFR), have been identified as substrates for ubiquitination.

RTKs are single pass transmembrane proteins that control a wide variety of cellular events in pluricellular organisms including cell proliferation, cell differentiation, cell migration, and cell survival. In normal cells, RTK activation is tightly regulated. Their inappropriate activation is associated with the development and progression of many human malignancies. Of the 58 genes known to encode RTKs, the deregulation of 30 has been associated with human tumors (Blume-Jensen and Hunter, 2001). In the past two decades, several mechanisms that deregulate RTKs, such as receptor amplification, chromosomal translocation, and point mutations, have been identified. These changes result in ligand-independent activation or enhanced catalytic activity of RTKs (Blume-Jensen and Hunter, 2001; Lamorte and Park, 2001). However, in addition to these positive mechanisms, there is growing evidence that escape from negative regulatory mechanisms is an important event in RTK deregulation. In this review, we will examine how different RTK-derived oncoproteins escape downregulation.

In the absence of ligand, most RTKs are catalytically inactive. Binding of the ligand promotes receptor dimerization/oligomerization and induces a conformational change that triggers receptor kinase activity. Tyrosine residues on the receptor then become phosphorylated, forming binding sites for proteins that relay the biological signals. RTK activation promotes their internalization via clathrin-coated pits. The downregulation of tyrosine kinase activity and hence signaling can be modulated reversibly through the action of tyrosine phosphatases as well as irreversibly through their lysosomal degradation. Recent publications have established that ubiquitination plays a major role in RTK downregulation by targeting receptors to the lysosome (Shtiegman and Yarden, 2003).

Protein ubiquitination is mediated by an enzymatic cascade composed of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3). A single 76 amino acid ubiquitin moiety is covalently conjugated to a lysine residue within the substrate protein. Additional ubiquitin residues can be added to lysine residues within the linked ubiquitin to generate polyubiquitin chains. The presence of a polyubiquitin chain on many cytosolic and nuclear proteins targets them for degradation by the 26S proteasome. In contrast, ubiquitination of many cell surface receptors correlates with their internalization and lysosomal degradation. Monoubiquitination of EGFR is sufficient for its internalization, and some evidence supports that EGFR and PDGFR are multimonoubiquitinated rather then being polyubiquitinated (Haglund et al., 2003; Mosesson et al., 2003).

Monoubiquitin moieties can constitute binding sites for proteins that contain ubiquitin binding domains such as ubiquitin interaction motif (UIM), ubiquitin-associated (UBA), and ubiquitin-conjugating-like (UBC-like) domains. The UIM-containing proteins, HRS and STAM, are thought to recruit ubiquitinated RTKs to ESCRT complexes that retain receptors in specialized microdomains of sorting endosomes characterized by a bilayered clathrin coat, from which receptors internalize into the endosomal lumen (Figure 1) (Clague, 2002). Sorting endosomes mature into multivesicular bodies (MVBs) where receptors remained trapped within internal vesicles. Fusion of MVBs with lysosomes leads to the degradation of internal vesicles and their content. RTKs that are not ubiquitinated are not sorted in the bilayered clathrin coat, can recycle back to the cell surface, and escape lysosomal degradation (Figure 1) (Katzmann et al., 2002).

The Cbl family of ubiquitin-protein ligases (c-Cbl, Cbl-b, and Cbl-3) plays a major role in the ligand-dependent ubiquitination of many RTKs (Thien and Langdon, 2001). Several receptors including EGFR, PDGFR, CSF-1R, and Met (HGFR) are ubiquitinated following recruitment of c-Cbl. Cbl ubiquitin-protein ligases are modular proteins that contain a conserved N-terminal tyrosine kinase binding (TKB) domain and a RING finger domain in addition to other protein interaction motifs (Thien

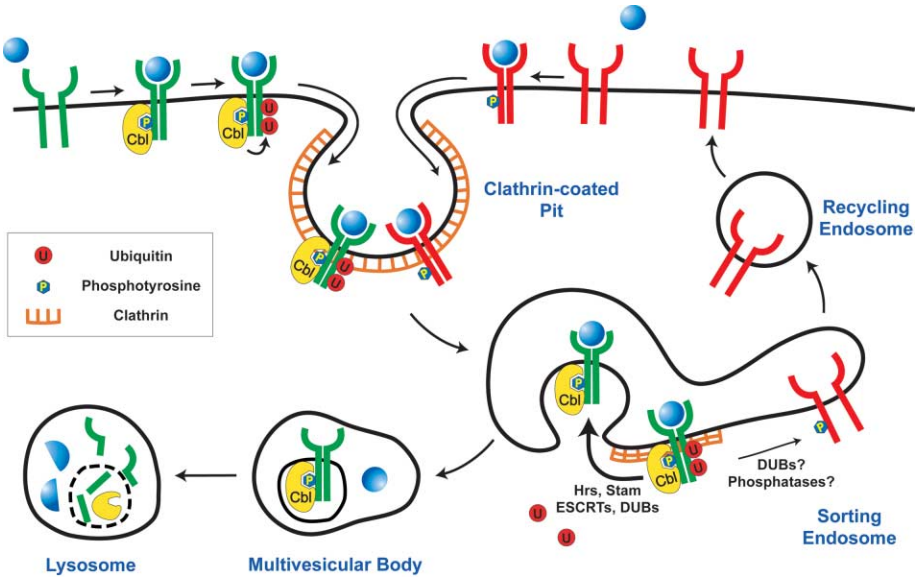

Figure 1. RTK ubiquitination and downregulation

Subsequent to the activation of several RTKs, c-Cbl is recruited to the receptor and induces receptor ubiquitination. Following internalization, ubiquitinated RTKs (green) are enriched in an endosomal microdomain characterized by a bilayered clathrin coat. Receptors are subsequently internalized in inner vesicles. The process of receptor enrichment and subsequent internalization involves multiple proteins that contain ubiquitin-interacting motifs (Hrs, Stam, and proteins of the ESCRT complexes) as well as deubiquitinating enzymes (DUBs) that remove ubiquitin moieties from the receptors. Fusion of multivesicular bodies with lysosomes leads to the degradation of inner vesicles and their content by lysosomal proteases. RTKs that are not ubiquitinated (red) are not sequestered in the bilayered clathrin coat of the sorting endosomes and can be recycled to the cell surface where they can be reactivated. Such receptors may be inactivated through dephosphorylation.

and Langdon, 2001). Where tested, the TKB domain interacts with specific phosphotyrosine residues on RTKs. The RING finger domain recruits the E2 ubiquitin-conjugating enzyme, UbcH7. Both domains are required for the transfer of ubiquitin residues to RTKs. Moreover, the stability of c-Cbl is itself regulated by ubiquitination. Tyrosine phosphorylation of c-Cbl by the c-Src protein tyrosine kinase promotes auto-ubiquitination of c-Cbl and its degradation in a proteasome-dependent manner (Yokouchi et al., 2001). The observation that Cbl proteins can also interact with ubiquitin-protein ligases containing HECT (*h*omologous to *E*6-AP *C t*erminus) domains suggests a potential additional layer of regulation (Courbard et al., 2002).

In addition to the targeting of RTKs for lysosomal degradation following ubiquitination, several lines of evidence support a role for c-Cbl in the internalization of RTKs. The overexpression of c-Cbl enhances the rate of internalization of EGFR (Levkowitz et al., 1999; Soubeyran et al., 2002), whereas in c-Cbl null macrophages, CSF-1R has a slower rate of internalization (Lee et al., 1999). It has been proposed that c-Cbl promotes the internalization of EGFR and the Met receptor through its ability to recruit CIN85 and endophilins (Petrelli et al., 2002; Soubeyran et al., 2002). During RTK internalization, endophilins are thought to induce negative membrane curvature required for the invagination of the plasma membrane into pits.

Consistent with its role in the downmodulation of RTKs, mutant c-Cbl proteins that lack ubiquitin ligase activity have been identified in mouse tumors and as retrovirally transduced transforming proteins (Thien and Langdon, 2001). These proteins retain the ability to bind to phosphotyrosine residues on RTKs, yet fail to ubiquitinate the receptor. Hence, they are thought to compete with the binding of wt Cbl proteins to RTKs and to transform cells through their ability to potentiate RTK signals.

Recently, it has become evident that several oncogenic RTKs have lost the ability to recruit Cbl in a TKB-mediated manner (Met/HGFR, CSF-1R, c-Kit/SCFR, EGFR). These receptors normally recruit Cbl following ligand stimulation, and for several of them, Cbl has been shown to promote receptor ubiquitination and enhanced degradation.

Hepatocyte growth factor receptor

The c-Cbl TKB domain binds to a juxtamembrane tyrosine (1003) residue on the Met receptor, and this interaction is essential for ubiquitination and degradation of the Met receptor (Peschard et al., 2001). An oncogenic form of the Met receptor, Tpr-Met, was generated following a carcinogen-induced chromosomal rearrangement that fused a protein dimerization domain (Tpr) to the kinase domain of the Met receptor. This results in the deletion of the juxtamembrane tyrosine binding site for c-Cbl (Y1003). The Tpr-Met RTK oncoprotein is constitutively activated, but fails to bind c-Cbl and is not ubiquitinated (Figure 2) (Peschard et al., 2001). This suggested that loss of Cbl recruitment and ubiquitination contribute to the oncogenic deregulation of Tpr-Met. Notably, a Met receptor mutant that lacks only the c-Cbl TKB domain binding site (Y1003F) has a prolonged half-life and is oncogenic in cell culture and tumorigenesis assays, identifying c-Cbl and ubiquitination as important negative regulators for this receptor (Peschard et al., 2001). Another member of the Met family of RTKs, c-Sea, was originally isolated as the cellular homolog of the avian erythroblastosis retroviral oncoprotein v-Sea. Like Tpr-Met, the c-Cbl TKB binding site located in the juxtamembrane domain of c-Sea is deleted in the v-Sea oncogenic receptor (Figure 2).

Colony-stimulating factor-1 receptor

In a similar manner, the CSF-1R was first identified as a viral oncogene, v-Fms, the product of the McDonough strain of feline sarcoma virus. One of the several structural differences between c-Fms/CSF-1R and v-Fms is the replacement of the CSF-1R carboxy-terminal 50 amino acids that contain the direct binding site for the c-Cbl TKB domain (tyrosine 969) by 14 unrelated amino acids in v-Fms (Figure 2) (Mancini et al., 2002; Wilhelmsen et al., 2002). The deletion of the c-Cbl direct binding site in v-Fms renders v-Fms refractory to c-Cbl-dependent ubiquitination, and the addition of the c-Cbl TKB domain binding site to v-Fms decreases its transforming activity (Mancini et al., 2002). Consistent with these observations, mutation of the C-terminal tyrosine 969, the direct binding site for c-Cbl, enhanced the transforming ability of CSF-1R in fibroblasts (Roussel et al., 1988). Mutations of the Cbl binding site are frequently observed

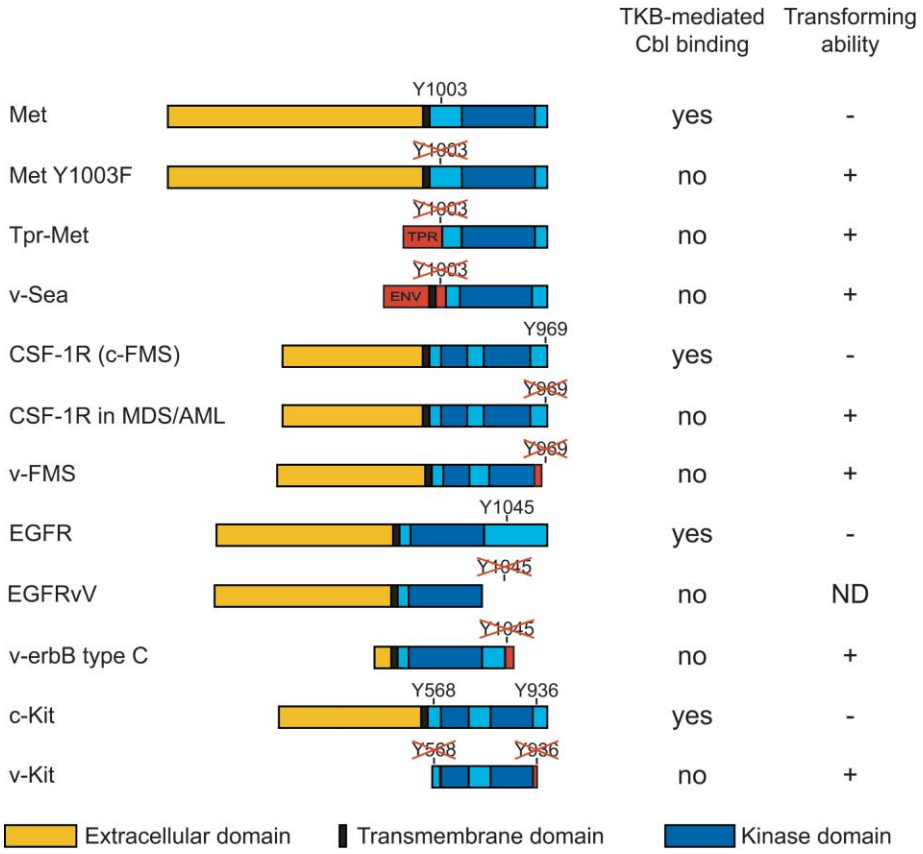

Figure 2. Loss of TKB-mediated Cbl binding in several RTK-derived oncoproteins

The binding of the c-Cbl TKB domain to the EGF, CSF-1, and Met receptors is required for receptor ubiquitination and degradation. Loss of TKB-mediated Cbl binding to RTKs correlates with a gain of transforming ability, suggesting that it constitutes an important event in RTK oncogenic deregulation.

in CSF-1R in human myelodysplasia (8 out of 67 cases) and acute myeloblastic leukemia (8 out of 48 cases), further implicating loss of c-Cbl binding in oncogenic deregulation of CSF-1R in human cancer (Ridge et al., 1990).

Stem cell factor receptor, c-Kit

The recruitment of c-Cbl to the stem cell factor (SCF) receptor/c-Kit and to PDGFRβ occurs indirectly via the APS (*a*daptor containing *P*H and *S*H2 domains) protein. This association leads to PDGFR downregulation (Yokouchi et al., 1999). Notably, the two APS binding sites in c-Kit, tyrosine 568 located in the juxtamembrane domain and tyrosine 936 located in the carboxy-terminal region of the receptor (Wollberg et al., 2003), are absent in v-Kit, the transforming protein of the Hardy-Zuckerman-4 strain of feline sarcoma virus (Figure 2). Moreover, the deletion of APS binding sites in c-Kit greatly enhanced its transforming ability (Herbst et al., 1995), although some of this may be attributed to the loss of negative regulation through juxtamembrane tyrosine 568 and enhanced catalytic activity of the receptor (Chan et al., 2003).

Epidermal growth factor receptor

Upon activation of EGFR (ErbB1), c-Cbl proteins are rapidly recruited and remain associated with the receptor as it progresses through the endocytic pathway (de Melker et al., 2001). Tyrosine 1045 constitutes the direct binding site for the c-Cbl TKB domain and is required for c-Cbl-mediated ubiquitination and degradation of EGFR (Levkowitz et al., 1999). Interestingly, the *v-erbB* protein encoded by the avian erythroblastosis virus AEV-C has, among other alterations, an internal deletion of 21 amino acids that comprises the c-Cbl TKB domain binding site (Figure 2) (Choi et al., 1986). Moreover, in a similar manner to both HGF and CSF-1 receptors, an EGFR mutant lacking only the direct Cbl binding site elicits stronger mitogenic signals than the wt receptor (Waterman et al., 2002).

Up to 40% of glioblastomas express oncogenic mutants of EGFR. The most common genetic alteration consists of a deletion of exons 2–7 located in the extracellular domain of EGFR (67% of all EGFR genetic alterations) (Frederick et al., 2000). The resulting protein (EGFRvIII) is constitutively activated, but has low levels of tyrosine phosphorylation. EGFRvIII receptors have a reduced ability to recruit Cbl proteins and CIN85, and are neither ubiquitinated nor internalized (Schmidt et al., 2003), suggesting that basal receptor activation is sufficient to confer tumorigenicity, but not sufficient to trigger Cbl-mediated downregulation. Another genetic alteration of EGFR identified in human glioblastoma generates a truncated receptor (EGFRvV) that has an intact kinase domain, but that is missing the c-Cbl TKB direct binding site (Y1045) and the internalization signals (Figure 2) (Frederick et al., 2000).

Another member of the EGF receptor family, HER2/ErbB2, is overexpressed in many human tumors such as breast, ovary, prostate, and brain tumors. Overexpression of HER2 shifts the formation of EGFR homodimers toward the formation of EGFR/HER2 heterodimers. While ligand-stimulated EGFR homodimers undergo rapid ubiquitination, internalization, and degradation, EGFR/HER2 heterodimers recruit c-Cbl to a reduced extent, are slowly internalized, and recycle rapidly to the cell surface (Lenferink et al., 1998; Muthuswamy et al., 1999). The delay in EGFR/HER2 heterodimer degradation potentiates EGFR-dependent cell proliferation, cell migration, and antiapoptotic signals. Hence, the overexpression of HER2 constitutes a mechanism through which EGFR escapes Cbl-mediated downregulation. Indeed, overexpression or amplification of Cbl binding RTKs, as frequently observed in human cancers, could act as a mechanism to sequester Cbl proteins, enhancing the stability of other RTKs that are Cbl substrates. In addition, the deregulation of Src, which occurs in human colon, breast, lung, and brain cancers, leads to the stabilization of the EGFR by promoting c-Cbl ubiquitination and degradation (Bao et al., 2003).

Besides point mutations and overexpression, RTKs are frequently activated in human tumors following chromosomal translocation. In general, this fuses a protein dimerization domain with the cytosolic kinase domain of the receptor, resulting in constitutive receptor dimerization and activation (Lamorte and Park, 2001). Over 25 RTK-derived fusion proteins have

been identified in human tumors. In each case, the N-terminal signal peptide, necessary for protein targeting to the membrane, is deleted in the rearranged kinase and, with the exception of FIG-ROS that is targeted to the golgi (Charest et al., 2003), where studied, these proteins are cytosolic (Lamorte and Park, 2001). Localization to the cytosol would preclude their entry in the endocytic pathway and hence, their lysosomal targeting and degradation. However, it remains to be determined whether these oncoproteins are ubiquitinated and targeted for degradation by the proteasomal pathway.

Conclusion

In the past few years, the discovery that Cbl proteins are ubiquitin-protein ligases and that ubiquitination regulates receptor sorting to lysosomes has greatly improved our understanding of the molecular mechanisms that downregulate RTKs. We bring to light evidence that many RTK-derived oncoproteins avoid downregulation by loss of Cbl binding sites, inefficient Cbl recruitment, Cbl degradation, or through the formation of fusion proteins that escape lysosomal degradation. These constitute some mechanisms among several that promote the stabilization of RTKs. Overall, an understanding of the mechanisms through which RTKs are downmodulated will allow new therapeutic approaches to target these RTKs for degradation in cancer. In this respect, Trastuzumab, a drug used in combination with chemotherapy for the treatment of metastatic breast cancer, is a weak agonist of HER2. Trastuzumab is thought to force receptor dimerization and potentiate recruitment of Cbl and receptor downregulation (Klapper et al., 2000). Similar drugs designed to force other RTKs toward a degradation pathway would likely be beneficial in the design of therapeutic strategies for cancer.

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