

Accelerated Publication

Endocytosis of Receptor Tyrosine Kinases Is Driven by Monoubiquitylation, Not Polyubiquitylation*

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Growth factors stimulate specific receptor tyrosine kinases, but subsequent receptor endocytosis terminates signaling. The ubiquitin ligase c-Cbl targets epidermal growth factor receptors (EGFRs) to endocytosis by tagging them with multiple ubiquitin molecules. However, the type of ubiquitylation is unknown; whereas polyubiquitin chains signal proteasomal degradation, ubiquitin monomers control other processes. We report that in isolation c-Cbl mediates monoubiquitylation rather than polyubiquitylation of EGFRs. Consistent with the sufficiency of monoubiquitylation, when fused to the tail of EGFR, a single ubiquitin induces receptor endocytosis and degradation in cells. By using receptor and ubiquitin mutants, we infer that c-Cbl attaches a founder monoubiquitin to the kinase domain of EGFR and this is complemented by the conjugation of additional monoubiquitins. Hence, receptor tyrosine kinases are desensitized through conjugation of multiple monoubiquitins, which is distinct from polyubiquitin-dependent proteasomal degradation.

Protein ubiquitylation has emerged as a versatile regulatory strategy (reviewed in Ref. 1). In its best characterized role as a signal for proteasomal degradation, productive recognition of ubiquitylated substrates is shown to minimally require a tetra-ubiquitin chain (2). Alternatively, studies in yeast attribute to monoubiquitylation an intrinsic capacity to target substrates both for internalization at the plasma membrane and sorting at multivesicular bodies toward destruction in the vacuole (reviewed in Ref. 3). With subsequent identification of ubiquitin binding activities, such as the UIM,¹ a rationale for ubiquitin-

dependent recognition of substrates has materialized (4–7). In higher eukaryotic systems, ubiquitylation of cell-surface receptors, likewise, correlates with their down-regulation via orthologous trafficking pathways that employ counterparts conserved from yeast (8–11). Ligand-activated ubiquitylation of EGFR, as well as other RTKs, is mediated by c-Cbl (12–14). Whether or not EGFR ubiquitylation is sufficient for its internalization remains an open question. Likewise, although it is clear that each endocytosed receptor is conjugated to several molecules of ubiquitin, it is currently unknown to which extent branching of the EGFR-conjugated ubiquitins occurs in living cells. Here we present evidence indicating that the action of c-Cbl is limited to the addition of monomeric ubiquitins, and these are sufficient for receptor endocytosis and degradation.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Unless indicated, reagents were purchased from Sigma. E1 was from Affiniti (Mamhead, Exeter, UK), and rabbit reticulocyte lysate from Promega (Madison, WI). The 528-IgG antibody was isolated from hybridomas and a Fab fragment prepared and labeled with Cy3. An antibody to EGFR was from Alexis (San Diego, CA). Anti-EEA1 mouse antibody was from Transduction Laboratories (Lexington, KY). Fluorescently labeled antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Construction of Expression Vectors—A plasmid encoding a truncated EGFR (EGFR-ΔC) was prepared by introducing a STOP codon after amino acid 1087. The EGFR-ΔC4R mutant was generated by site-directed mutagenesis. Vectors expressing EGFR::Ub chimeras were prepared by overlap extension PCR and mutations introduced. HA-tagged ubiquitin, either WT or K0, was subcloned into pEFIREs. Bacterial expression vectors for wild type (His)₆HA-Ub and (His)₆HA-Ub-K0 were obtained by subcloning into the pET28 plasmid (Novagen). Recombinant ubiquitins were subsequently affinity-purified on Ni²⁺-conjugated agarose beads.

Transient Transfection, Immunoprecipitation, and Immunoblotting—Transfections were carried out using 1 μg of DNA of each expression vector, and the total amount of DNA normalized with the respective empty plasmid. Cells were assayed 48 h after transfection. Whole cell lysates were analyzed by SDS-PAGE, either directly or after immunoprecipitation, and protein bands detected with an enhanced chemiluminescence reagent.

In Vitro Ubiquitylation Assay—Receptor immunoprecipitates were extensively washed and resuspended in ubiquitylation buffer (40 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, and 2 mM dithiothreitol) supplemented with 2 mM ATP, and containing either rabbit reticulocyte lysate (1 μl) or recombinant E1 (0.1 μg) and E2 (UbcH5C; 7 μl of crude bacterial extract). Wild type or mutant (His)₆HA-tagged ubiquitin (0.5 μg) and GST-Cbl (0.1 μg) were added as indicated. Reaction mixtures were incubated for 1 h at 30 °C.

Immunofluorescence—Cy3-labeled Fab (20 μg/ml) was incubated with cells at 4 °C for 90 min. Thereafter, cells were transferred to 37 °C for the indicated time intervals before fixation and visualization. Alternatively, paraformaldehyde-fixed cells were permeabilized for 10 min at 22 °C with phosphate-buffered saline containing 1% albumin and 0.2% Triton X-100. For labeling, cells were incubated for 1 h with an anti-EGFR antibody and after extensive washes incubated for an additional hour with a Cy3-conjugated donkey anti-mouse F(ab)₂. Alternatively, coverslips were co-incubated with monoclonal anti-HA and anti-EEA1 antibodies, washed thoroughly, and incubated with a Cy3-conjugated donkey anti-rat F(ab)₂ and a Cy2-conjugated donkey anti-mouse F(ab)₂.

RESULTS

In Cells c-Cbl Promotes Conjugation of Several Ubiquitins to Each Receptor Molecule, but in Isolation It Appends Monomeric Ubiquitins to EGFRs—In line with previous reports, when

kinase; WT, wild type; GST, glutathione S-transferase; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.

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¹ The abbreviations used are: UIM, ubiquitin-interacting motif; CHO, Chinese hamster ovary; EGFR, epidermal growth factor receptor; HA, hemagglutinin; Ub-K0, a lysine-less ubiquitin; RTK, receptor tyrosine

co-expressed with EGFR in CHO cells, c-Cbl destabilized the receptor and enhanced receptor ubiquitylation (Fig. 1A). When immunoblotted, the destabilized EGF-activated receptors ex-

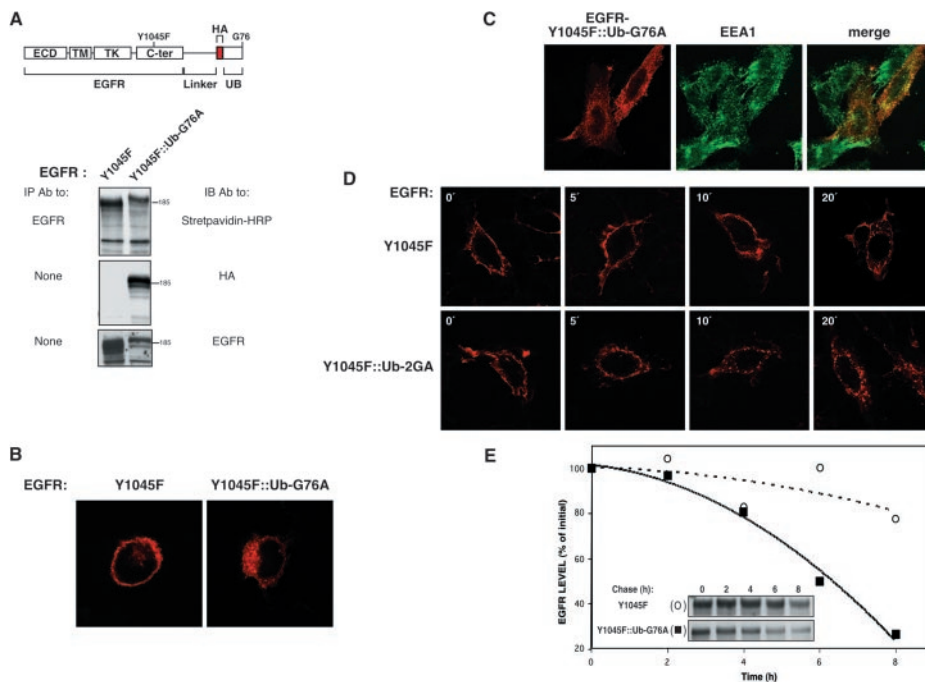
hibited a ladder of bands representing molecules differing in the number of conjugated ubiquitins. This stoichiometric multiplicity can be attributed to conjugation of either a ubiquitin polymer (henceforth, polyubiquitylation), many monomeric ubiquitins attached to several receptor's lysines (henceforth, multiubiquitylation), or a combination of mono- and polyubiquitins.

Polymerization of ubiquitin on a substrate utilizes certain lysines as branching sites (reviewed in Ref. 1). Therefore, to determine whether c-Cbl possesses mono- or polyubiquitylating activity, we compared the *in vitro* modifying capacity of wild type ubiquitin (WT-Ub) with that of Ub-K0 that, by definition, is unable to form polymeric chains. Both forms were expressed in bacteria and included as the sole source of ubiquitin in reconstituted reactions containing an isolated EGFR. Surprisingly, upon co-incubation with a bacterially expressed c-Cbl, both WT-Ub and Ub-K0 reproduced similar patterns that, according to the observed molecular shift, corresponded primarily to a monoubiquitylated EGFR (Fig. 1B). Hence, the results suggest that c-Cbl is equipped with an intrinsic monoubiquitylating, rather than polyubiquitylating, activity.

An Internalization-defective Mutant of EGFR Acquires Rapid Endocytosis and Degradation When Fused to a Single Ubiquitin—We predicted that once EGFR is tagged by a single molecule of ubiquitin, it will be sorted for endocytosis even when coupling to c-Cbl is not permitted. To test this prediction we utilized an internalization-defective mutant of EGFR, incapable of direct c-Cbl binding (EGFR-Y1045F (15)). A single copy of ubiquitin was fused to the carboxyl terminus of Y1045F-EGFR, but to prevent formation of covalent adducts, we replaced the terminal glycine (Ub-G76), or both glycine 75 and glycine 76 (Ub-2GA) with alanines. Cell-surface biotinylation assays confirmed maturation and delivery of both parental and chimeric receptor forms to the plasma membrane (Fig. 2A). In unstimulated cells EGFR-Y1045F is not ubiquitylated (15) and, consistent with previous studies, was found predominantly at the plasma membrane (Fig. 2B). In contrast, EGFR-Y1045F::Ub-G76A localized primarily to structures resembling endosomes. Indeed, co-staining of EGFR-Y1045F::Ub-G76A and the early endosomal marker, EEA1 (16), revealed a highly significant degree of co-localization (Fig. 2C).

FIG. 1. In cells c-Cbl promotes conjugation of several ubiquitins to each EGFR molecule, but in isolation only monomers of ubiquitin are conjugated by c-Cbl. A, CHO cells expressing EGFR, HA-Ub, and c-Cbl, as indicated, were incubated without or with EGF (100 ng/ml) for 15 min at 37 °C, and cell lysates were analyzed with the indicated antibodies. B, EGFR isolated from untreated A431 cells was subjected to an *in vitro* ubiquitylation in the presence of E1 and E2 (UbcH5C), together with (His)₆HA-Ub, WT or K0, and GST-Cbl, as indicated. *pTyr*, phosphotyrosine; *IB*, immunoblotting; *Ab*, antibody; *IP*, immunoprecipitation.

FIG. 2. Covalently attached monoubiquitin is sufficient to promote endocytosis and degradation of EGFR. A: *top*, representation of EGFR-Y1045F::Ub; *bottom*, EGFR-expressing CHO cells were surface-biotinylated on ice and analyzed with the indicated antibodies. B, EGFR-expressing CHO cells were fixed, permeabilized, and incubated with an anti-EGFR antibody, followed by a Cy3-labeled secondary antibody. C, cells expressing EGFR-Y1045F::Ub-G76A were fixed, permeabilized, and co-incubated with antibodies against EEA1 and HA. Secondary fluorescent antibodies were used for detection. D, HeLa cells expressing the indicated EGFR forms were preincubated for 90 min on ice with a Cy3-labeled 528-Fab. Subsequently, cells were incubated at 37 °C for the indicated intervals, fixed, and analyzed. E, CHO cells expressing EGFR-Y1045F (*circles*) or EGFR-Y1045F::Ub-G76A (*squares*) were subjected to metabolic labeling with ³⁵S-labeled amino acids for 12 h. EGFR was immunoprecipitated following the indicated chase intervals. Shown are average decay curves and an autoradiogram of a representative experiment. *IB*, immunoblotting; *IP*, immunoprecipitation; *ECD*, extracellular domain; *TM*, transmembrane domain; *TK*, tyrosine kinase; *C-ter*, carboxyl-terminal; *HRP*, horseradish peroxidase; *Ab*, antibody.



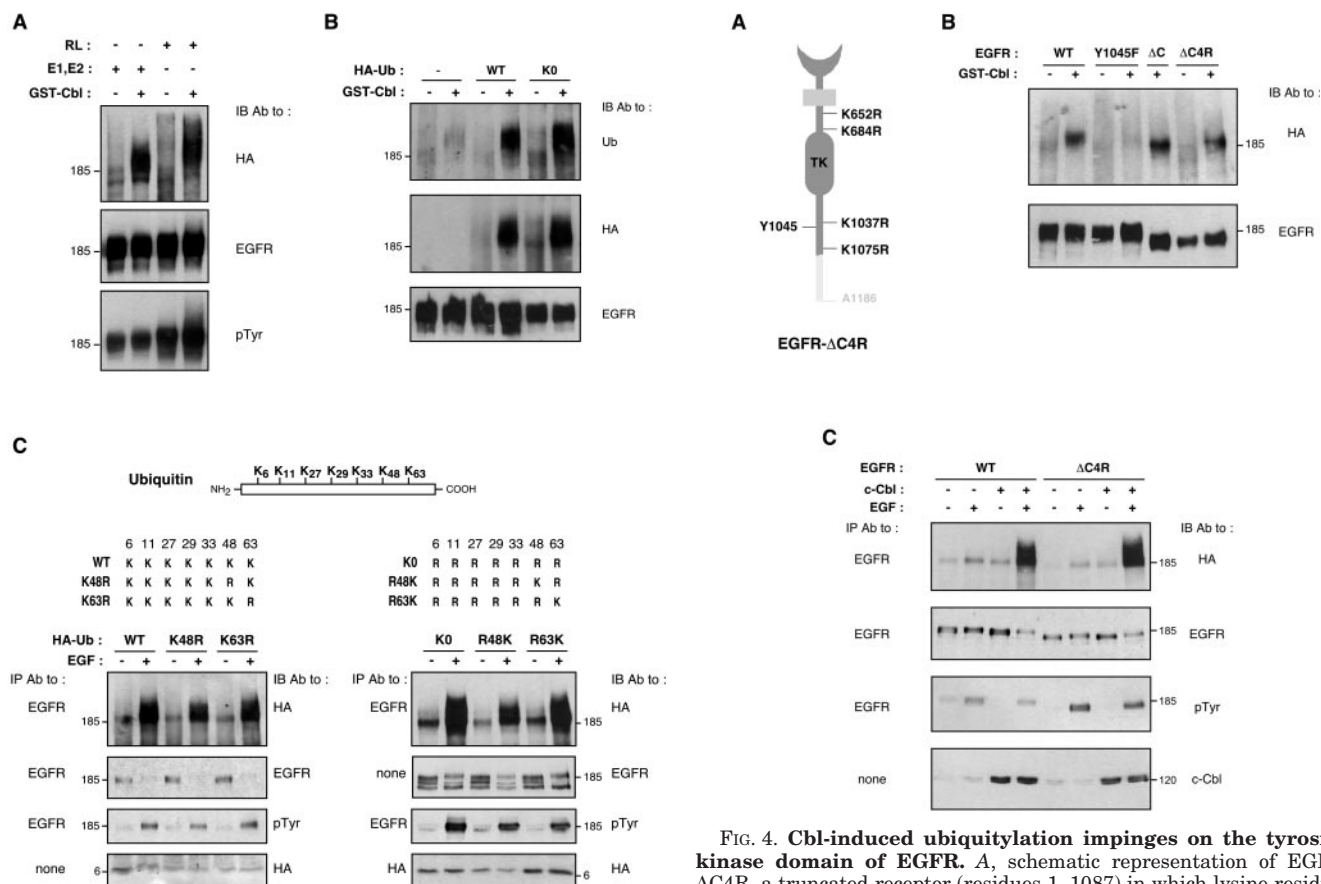


FIG. 3. EGFR undergoes multiubiquitylation, not polyubiquitylation. *A*, EGFR immunoprecipitates from untreated A431 cells were ubiquitylated *in vitro* in the presence of either recombinant E1 and E2, or reticulocyte lysate (RL), together with (His)₆HA-Ub and GST-Cbl, as indicated. Reaction mixtures were incubated at 30 °C for 60 min before immunoblotting. *B*, *in vitro* ubiquitylation of isolated EGFRs was performed in the presence of reticulocyte lysate. GST-Cbl and HA-Ub were added as indicated. *C*: top, representation of all lysines of ubiquitin. Forward (lysine-to-arginine) and add-back (arginine-to-lysine) mutants are depicted. Bottom, CHO cells expressing c-Cbl and EGFR, along with the indicated forms of HA-Ub, were incubated without or with EGF for 15 min at 37 °C, and cell lysates were analyzed as indicated. *pTyr*, phosphotyrosine; *IB*, immunoblotting; *IP*, immunoprecipitation; *Ab*, antibody.

To track endocytosis of EGFR and yet avoid ligand- or antibody-induced internalization, we used a fluorescently labeled monovalent fragment of an anti-EGFR antibody (528-Fab). First, this analysis left out the possibility that EGFR:Ub chimeras, like some lysosomal enzymes (5, 6), reach endosomal structures directly from the biosynthetic pathway. Second, when cells were preincubated on ice with 528-Fab and then transferred to 37 °C, we noted different kinetics of internalization; unlike EGFR-Y1045F, which remained largely at the cell surface and started appearing in intracellular vesicles only after 20 min (Fig. 2D), some chimeric receptors translocated into endosomes already 5 min after transfer to 37 °C, and their endocytosis peaked at 10 min. In line with different endocytic behavior, metabolic labeling showed the chimeras to be degraded considerably more rapidly than EGFR-Y1045F in the absence of EGF (Fig. 2E). These results imply that monoubiquitylation is sufficient for internalization of EGFR, and together with the data presented in Fig. 1, they suggest that Cbl-mediated tagging of monomeric ubiquitins sorts active receptors to degradation.

Decoration of EGFR with Monoubiquitins, Not Polyubiquitins, Is Sufficient for Ligand-induced Receptor Degradation—

FIG. 4. Cbl-induced ubiquitylation impinges on the tyrosine kinase domain of EGFR. *A*, schematic representation of EGFR-ΔC4R, a truncated receptor (residues 1–1087) in which lysine residues not included within the tyrosine kinase (TK) domain were mutated. *B*, EGFRs derived from unstimulated transfected HEK-293T cells were subjected to ubiquitylation *in vitro* in the presence of recombinant E1 and E2. *C*, CHO cells expressing HA-Ub, c-Cbl, and either wild type EGFR (WT) or the truncation mutant (ΔC4R), were analyzed as indicated. *pTyr*, phosphotyrosine; *IB*, immunoblotting; *IP*, immunoprecipitation; *Ab*, antibody.

The observed difference between c-Cbl's activity in isolation (monoubiquitylation) and in cells (multi- or polyubiquitylation) implies recruitment of a collaborating activity. Hence, we compared ubiquitylation of EGFR in the presence of recombinant E1 and E2 or a crude mixture of cellular factors (namely, reticulocyte lysate). Analysis under electrophoretic conditions aimed at resolving ubiquitylated species detected a significantly higher ladder of EGFR when incubated with c-Cbl and reticulocyte lysate (Fig. 3A). The smeary pattern was particularly detectable by anti-phosphotyrosine antibodies, indicating selectivity to active receptors. Furthermore, replacing wild type ubiquitin with a Ub-K0 did not change the ubiquitylation pattern (Fig. 3B). Conceivably, Cbl-induced monoubiquitylation of phosphorylated EGFRs is followed by recruitment of unknown effectors, which mediate additional monoubiquitylation.

To resolve whether EGFR ubiquitylation in cells entails monomeric or polymeric ubiquitin, we utilized a series of ubiquitin mutants with individual lysine-to-arginine substitutions at known sites for chain branching *in vivo* (lysines 11, 29, 48, and 63 (1, 17)). Preliminary analyses indicated that the ectopic ubiquitin attained a >10-fold excess over the endogenous molecule 18 h after transfection. Under these conditions all four mutants, namely K11R, K29R, K48R, and K63R, reconstituted similar patterns of ligand-induced EGFR ubiquitylation as wild-type ubiquitin (Fig. 3C and data not shown). Furthermore, no mutant inhibited or delayed EGF-induced degradation of EGFR, and hence, none of the tested lysines appears to be

involved in ubiquitin chain branching. To consolidate this conclusion, reciprocal experiments were performed using Ub-K0. When overexpressed, this mutant acts as a terminator of ubiquitin polymerization. However, Ub-K0 expressing cells effectively incorporated the mutant form of ubiquitin into EGFR molecules, which retained their normal ubiquitylation pattern (Fig. 3C). As expected, when tested in conjunction with β -catenin, a well characterized substrate of polyubiquitylation and proteasomal degradation, Ub-K0 abolished the typical ladder of ubiquitylated β -catenin (data not shown). Moreover, add-back mutants derived from Ub-K0 underwent comparable conjugation to EGFR, and even though they limited the extent of receptor degradation compared with WT-Ub, none extended or enhanced the ladder of ubiquitylated EGFRs (Fig. 3C and data not shown). In conclusion, because all lysine mutants of ubiquitin coherently generated a pattern consistent with multiubiquitylation, these results reinforce a role for monoubiquitin in sorting EGFR to endocytosis.

Multiubiquitylation Confined to the Kinase Domain of EGFR Is Sufficient for Receptor Degradation—The results presented suggest that multiubiquitylation of EGFR is preceded by a monoubiquitylating event. In an attempt to map the putative site, we generated a truncation mutant containing lysines only within its kinase domain (EGFR- Δ C4R; Fig. 4A), a region essential for c-Cbl recruitment (12). *In vitro*, EGFR- Δ C4R, like wild-type EGFR, underwent c-Cbl-mediated monoubiquitylation (Fig. 4B), raising the possibility that the kinase domain may be specifically targeted by c-Cbl. Testing EGFR- Δ C4R in living CHO cells lent support to this assignment. First, the EGFR- Δ C4R mutant, like the wild type receptor, underwent weak monoubiquitylation in unstimulated cells (Fig. 4C). Furthermore, regardless of its smaller number of potential acceptor sites, EGFR- Δ C4R underwent EGF-induced multiubiquitylation, and its degradation was accelerated by EGF and c-Cbl (Fig. 4C). Subsequent kinetic experiments, which are not presented, showed that both forms of EGFR underwent comparably rapid ubiquitylation, in support of the possibility that a kinase domain lysine serves as a founder monoubiquitylation site.

DISCUSSION

Fusion of a single ubiquitin to different integral membrane proteins led to the conclusion that ubiquitylation controls cargo endocytosis (11, 18, 19). Our work extends this notion to RTKs and envisages a stepwise process leading to the termination of growth factor signaling: ligand-induced phosphorylation of EGFR recruits c-Cbl (15), and then an E2 molecule, which is physically attached to c-Cbl, discharges its thioester-bonded ubiquitin at a lysine residue, likely located within the kinase domain of EGFR (Fig. 4). Because in isolation c-Cbl conjugates monomeric ubiquitins (Fig. 1B), and covalent attachment of a single ubiquitin drives endocytosis of an internalization-defective receptor (Fig. 2), we propose that the founder ubiquitin undergoes no branching, and its conjugation instigates sorting of ubiquitylated EGFRs. Predictably, the sorting mechanism involves adaptors bearing UIMs, such as Eps15 and epsin (7,

10). Conceivably, while at the cell surface or en route to the lysosome, EGFR is further decorated with additional monomers of ubiquitin. The mechanism of this secondary multiubiquitylation step remains unknown.

Considering the ability of a single ubiquitin to drive receptor endocytosis, it is worthwhile asking why EGFR is multi-ubiquitylated? For one, multiple monomers may confer resistance to inhibition by deubiquitylating enzymes. Alternatively, successive monoubiquitylation may increase the avidity of EGFR binding to adaptors like Eps15 (10). Last, multiubiquitylation rather than polyubiquitylation may confer to EGFR refractoriness to the 26S proteasome. Notably, both endocytic adaptors (20) and one of the ubiquitin-binding proteasomal subunits (21) utilize UIMs to recognize their cargoes and substrates. Hence, in the endosomal pathway, the intrinsic inability of c-Cbl to attach more than one ubiquitin to EGFR and to subsequently polymerize ubiquitin may suffice the endocytic machinery while evading degradation by proteasomes. Consistent with a mechanism that evades the proteasome, c-Cbl undergoes self-ubiquitylation with no associated proteasomal degradation (22). Hence, the E3 ligase activity of c-Cbl may be confined to monoubiquitylation. This issue and the possibility that other E3 ligases complement the action of c-Cbl toward the EGFR are matters for future investigation.

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