Conjugation to Nedd8 Instigates Ubiquitylation and Down-regulation of Activated Receptor Tyrosine Kinases*

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When appended to the epidermal growth factor receptor (EGFR), ubiquitin serves as a sorting signal for lysosomal degradation. Here we demonstrate that the ubiquitin ligase of EGFR, namely c-Cbl, also mediates receptor modification with the ubiquitin-like molecule Nedd8. EGF stimulates receptor neddylation, which enhances subsequent ubiquitylation, as well as sorting of EGFR for degradation. Multiple lysine residues, located within the tyrosine kinase domain of EGFR, serve as attachment sites for Nedd8. A set of clathrin coat-associated binders of ubiquitin also bind Nedd8, but they undergo ubiquitylation, not neddylation. We discuss the emerging versatility of the concerted action of ubiquitylation and neddylation in the process that desensitizes growth factor-activated receptor tyrosine kinases.

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‡ The abbreviations used are: EGFR, epidermal growth factor receptor; CHO, Chinese hamster ovary; GST, glutathione S-transferase; siRNA, small interference RNA; UIM, ubiquitin-interacting motif; APP-BP1, amyloid precursor protein-binding protein; ATPyS, adenosine 5′-O-(thiotriphosphate); E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; HA, hemagglutinin; Ub, ubiquitin.

Growth factors and their transmembrane receptors, harboring intrinsic tyrosine kinase activity, play essential roles in cell fate determination. Whereas the process leading to growth factor-induced activation of signaling pathways is relatively well understood, mechanisms that initiate signal desensitization are only beginning to be unraveled (reviewed in Ref. 1). In the case of the epidermal growth factor receptor (EGFR) and related receptor tyrosine kinases, the major signal attenuation process involves ligand-induced internalization of activated receptors and their sorting to degradation in lysosomes (reviewed in Ref. 2). In analogy to the pivotal role played by phosphotyrosine and respective binding domains (e.g. Src homology domain 2) in positive signaling pathways, ubiquitin and ubiquitin-binding domains (e.g. ubiquitin-interacting motifs (UIMs)) regulate receptor endocytosis and sorting for lysosomal degradation (3). For example, recruitment of an E3 ubiquitin ligase, called c-Cbl (4–7), enables subsequent conjugation of ubiquitin to multiple lysines of EGFR (8–10). The appended ubiquitins are thought to recruit a set of endocytic proteins (e.g. Eps15) through their UIMs (reviewed in Ref. 11).

In contrast to the well understood cellular functions of protein ubiquitylation, the roles played by ubiquitin-like proteins such as Nedd8 and SUMO are less characterized (12). Nedd8 is the closest kin of ubiquitin and it is linked to other proteins by an amide bond linking the carboxyl-terminal carboxylate to lysine residues. Neddylation is initiated by a heterodimeric complex comprising Uba3 and the amyloid precursor protein-binding protein (APP-BP1). Ubc12, an E2-like component, then mediates conjugation of Nedd8 (13), but the identity and function of Nedd8-specific E3 ligases are less understood. Modification of Cullins, the first discovered neddylation substrate, is promoted by Roc1/Rbx, a RING finger protein (14, 15), which led to the suggestion that Nedd8 assists the positioning of the E2-ubiquitin complex and subsequent ubiquitin transfer (21). Another target of neddylation is the cell cycle regulator, p53 (22). The present report identifies EGFR as a target for neddylation by c-Cbl. Receptor neddylation strictly depends on ligand stimulation, and in coordination with ubiquitin, it accelerates sorting of EGFR to degradation.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—Unless indicated, materials were purchased from Sigma, radioactive materials from Amersham Biosciences, and antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies to Nedd8 were from Alexis Biochemicals (San Diego, CA) or Boston Biochem (Cambridge, MA).

Receptor Down-regulation Assay—Ligand-induced down-regulation assays were performed as described (23). Briefly, cells grown in 24-well plates were incubated for up to 2 h with EGF (100 ng/ml) in binding buffer (Dulbecco’s modified Eagle’s/F-12 medium supplemented with 20 mM HEPES (pH 7.5) and 0.1% albumin) and then rinsed with ice-cold binding buffer. Surface-bound EGF was removed by using acetic acid wash (0.15 M NaCl, 0.15 M acetic acid, pH 2.8). The number of surface-exposed ligand binding sites was determined by incubating cells for 90 min at 4 °C with a radiolabeled EGF. Nonsp-
specific binding was measured in the presence of a 200-fold molar excess of the unlabeled ligand.

Receptor Internalization Assay—Ligand-induced internalization was measured as previously described (24). In brief, cells were incubated with $^{125}$I-EGF (10 ng/ml) for 2–10 min. Then, monolayers were rapidly washed with ice-cold binding buffer. Subsequently, surface-bound $^{125}$I-EGF was removed using a low pH acetic acid wash (pH 2.8) and quantified (surface-bound ligand). The cells were then solubilized and their radioactivity quantified (internalized ligand).

Construction of Expression Vectors and Transfection—Mutations were introduced using the QuikChange™ Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). A plasmid encoding a carboxyl-terminal FLAG-tagged EGFR was generated as follows. The FLAG sequence was inserted by PCR using oligonucleotides containing a BstEII site (5'-GCAGAAGGAGGC-AAATGC-3') or an Xhol site (5'-CAGATGCAGTGAATTT-ATGGTCAATCAGGAGGATTCAAGGACGAGGA-TAAGTAGCTGAGGCGG-3'). Ligation in an expression plasmid was performed following restriction of DNA encoding EGFR and FLAG with these enzymes. Human Ubc12 expression vector was received from R. L. Neve (Harvard Medical School). FLAG-Ubc12 was generated by PCR amplification of full-length Ubc12 and introduced in conjunction with a FLAG sequence into a pcDNA3 (Invitrogen). Mouse 3Myc-Nedd8 (25) was provided by S. Jentsch (Max Planck Institute for Biochemistry, Martinsried, Germany). Bacterial GST fusion proteins were constructed in pGEX (Amersham Biosciences). Cbl (4), GST-UIM (26), and ΔC4R-EGFR (8) expression vectors were previously described. Plasmids (1 μg, unless indicated) were introduced to cells using the Lipofectamine™ transfection method (Invitrogen). Unless otherwise indicated, cells were starved overnight for serum factors and then treated with EGF (100 ng/ml) for 15 min at 37 °C.

Establishment of Stably Expressing Chinese Hamster Ovary (CHO) Cell Derivatives—The CHO cell line carrying a temperature-sensitive E1 Nedd8-activating enzyme, ts41, and the parental cell line, E36, were obtained from J. Hirschberg (Hebrew University, Jerusalem). To establish stable derivatives expressing Nedd8, we co-transfected cells with pcDNA3-EGFR and pBabe-Puro vectors.

In Vitro Nedd8 Conjugation Assay—The reaction mixture contained rabbit reticulocyte lysate supplemented with 40 mm Tris-HCl (pH 7.5), 5 mm MgCl$_2$, 2 mm dithiothreitol, 2 mm ATP$_5$S, and $^{125}$I-labeled recombinant Nedd8. Bacterially purified recombinant GST-Cbl (50 μg) was added as indicated. EGFR was immunopurified from A431 cells and receptor-bound beads were incubated with the reaction mixture for 1 h at 30 °C. For labeling of Nedd8, we cleaved GST-Nedd8 with thrombin. Nedd8 (10 μg) was incubated with Na$^{125}$I (1 mCi) and the IODO-GEN reagent (Pierce). Following incubation at 22 °C (5 min), free iodine was separated from Nedd8 using the D-Salt Excellulose Desalting Column (Pierce).

Immunoprecipitation, Pulldown, and Immunoblotting Analyses—Cell extracts in solubilization buffer were cleared by centrifugation and incubated for 60 min at 4 °C with antibodies pre-coupled to immunoglobulin-agarose beads. The beads were washed three times, followed by addition of gel sample buffer. Samples were separated by electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked for 1 h in TBST buffer (20 mm Tris-HCl (pH 7.6), 0.15 m NaCl, 0.05% Tween 20) supplemented with albumin (3%), followed by blotting for 1 h with a primary antibody, washing with TBST and re-blotting with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected using a chemiluminescence reagent (Amersham Biosciences). For pulldown assays, cell lysates were incubated for 1 h at 4 °C with glutathione-agarose beads or with GST fusion proteins pre-coupled to glutathione-agarose beads.

Small Interfering RNA (siRNA)—The pSuper vector (27) was provided by R. Agami (The Netherlands Cancer Institute, Amsterdam). Nedd8-specific inserts that specify 19-nucleotide sequences based on the murine RNA were ligated in the BglIII and HindIII sites of pSuper. The following sense and the respective antisense sequence were selected for effective knockdown of Nedd8 in cells (construct C; nucleotides 162–181): 5'-GACAGCAGCTGATTACAAG-3'.

RESULTS

Multiple Nedd8 Molecules Conjugate to EGFR in a Ligand and c-Cbl-dependent Manner—UIMs recognize ubiquitylated cargo through a hydrophobic surface patch on ubiquitin (28). Because the corresponding patch is conserved in the ubiquitin-like protein Nedd8 and conjugation of the latter is limited to monomers, we hypothesized that Nedd8 plays a role in endocytosis of EGFR. Hence, we briefly stimulated HeLa or RWPE-1 prostate cells with EGF and probed anti-EGFR immunoprecipitates with antibodies specific to Nedd8. This analysis detected weak neddylation and rapid down-regulation of the endogenous EGFR (Fig. 1A). Similar results were obtained with receptor-null CHO cells ectopically expressing EGFR (Fig. 1A). Because c-Cbl ubiquitylates EGFR in a ligand-dependent manner, we tested if it can similarly conjugate Nedd8 to EGFR. Hence, we co-expressed EGFR and c-Cbl in CHO cells transfected with a plasmid encoding a peptide-tagged Nedd8. This experiment confirmed neddylation of EGFR upon treatment of cells with EGF (Fig. 1B).

To demonstrate that Nedd8 is directly conjugated to EGFR, rather than to an associated protein, we immunoprecipitated EGFR under denaturing conditions. This experiment made use of co-expressed FLAG-tagged EGFR, Myctagged Nedd8, and c-Cbl. Subsequent to stimulation with EGF, cell lysates were boiled in 1% SDS, prior to diluting out the detergent and immunoprecipitating EGFR. The enhanced signal observed upon stimulation with EGF, as well as the smeary appearance of the ligand-activated, covalently modified EGFR (Fig. 1C), indicate that Nedd8 directly conjugates to EGFR. In agreement with this conclusion, replacement of the carboxyl-terminal glycine of Nedd8 with an alanine prevented conjugation to EGFR (Fig. 1D). Furthermore, in line with the notion that Nedd8 forms no polymers analogous to lysine 48-linked chains of polyubiquitin, a mutant Nedd8 molecule whose lysine 48 has been replaced with an arginine retained normal conjugation to EGFR (Fig. 1D). Taken together with the retarded electro-
FIGURE 1. Multiple Nedd8 molecules are conjugated to EGFR in an EGF- and c-Cbl-dependent manner. A, the indicated cells were treated for 5 and 12 min with EGF and whole cell extracts were analyzed by immunoprecipitation (IP) and immunoblotting (IB) with anti-Nedd8 (Boston Biochem) or anti-EGFR antibodies, as indicated. Forty-eight hours post-transfection cells were treated for 15 min without or with EGF. Thereafter, whole cell extracts and EGFR immunoprecipitates were analyzed with the antibodies indicated. C, HeLa cells were co-transfected with plasmids encoding c-Cbl, FLAG-tagged EGFR, and Myc-tagged Nedd8. Forty-eight hours later, cells were incubated with EGF for 7 min, prior to cell lysis in solubilization buffer supplemented with SDS (1%) and boiling for 5 min. Thereafter, the SDS-containing solution was diluted (1:10) and EGFR immunoprecipitated using anti-FLAG antibodies. Following electrophoresis and transfer to a nitrocellulose filter, the filter was blotted with anti-Myc antibodies (upper panel), stripped, and re-blotted with an anti-EGFR antibody (lower panel). D, CHO cells were transiently transfected with vectors encoding EGFR and c-Cbl expression vectors, along with plasmids encoding the indicated Nedd8 mutants or an empty vector (H11002). Analyses were performed with the indicated antibodies. E, CHO cells were transfected with plasmids encoding EGFR and c-Cbl, along with the indicated amounts of a Myc-Nedd8 expression vector. Forty-eight hours post-transfection, cells were incubated without or with EGF for 15 min and cell lysates analyzed. F, CHO cells were transfected with an EGFR expression vector, along with an empty vector (closed circles), or vectors encoding Nedd8 (open circles), c-Cbl (closed triangles), or a combination of Nedd8 and c-Cbl plasmids (open triangles). Forty-eight hours after transfection, quadruplicate monolayers were incubated at 37 °C with EGF for the indicated time intervals. Thereafter, the surface receptor level was determined using a radiolabeled ligand-binding assay. Average and S.D. values (bars) are shown.
phoretic mobility of neddylated EGFR molecules, these observations imply that EGF enhances covalent conjugation of multiple (>10) monomers of Nedd8 to each receptor molecule.

Because the level of Nedd8 is dynamic (29), we examined the effect of Nedd8 expression levels on receptor degradation. The results presented in Fig. 1E demonstrate that gradually increasing Nedd8 expression, reciprocally affects EGF levels prior to activation by EGF, but the remaining receptor retains the characteristic ligand-induced degradation. Next, we examined the effect of ectopic Nedd8 in a receptor down-regulation assay. This assay utilizes transfected CHO cells, which overexpress EGF and therefore their down-regulation is limited, unless an exogenous c-Cbl is introduced (23, 30). As expected, ectopic expression of Nedd8 slightly accelerated the rate of EGF-induced down-regulation of EGFR (Fig. 1F). Likewise, ectopic expression of c-Cbl significantly enhanced receptor endocytosis, but maximal effects were observed upon co-expression of c-Cbl and an ectopic Nedd8 (Fig. 1F). In conclusion, c-Cbl mediates neddylation of EGFR, which is associated with accelerated removal of activated receptors from the cell surface.

The RING and TKB Domains of Cbl Are Necessary for Neddylation of EGFR

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The RING and TKB Domains of Cbl Are Necessary for Neddylation of EGFR—Phosphorylation of tyrosine 1045 of EGFR recruits the TKB domain of c-Cbl (4, 31). Hence, we examined a kinase-defective mutant of EGFR (K721A), as well as a receptor mutated at tyrosine 1045. As expected, neither mutant underwent neddylation in EGF-stimulated cells (Fig. 2A). Because the TKB domains of all three forms of Cbl, namely: c-Cbl, Cbl-b, and Cbl-3, share specificity, we envisaged that Cbl-b and Cbl-3 will mimic the neddylation effect of c-Cbl. This prediction was confirmed by ectopic co-expression of each Cbl protein together with Nedd8 (Fig. 2B and data not shown).

C-Cbl contains several domains, whose involvement in receptor neddylation was tested by analyzing a series of mutants (Fig. 2C). Deletion of 17 amino acids from the RING (70Z-Cbl) abolished neddylation of EGFR (Fig. 2C). Likewise, defecting the

FIGURE 2. The RING and TKB domains of Cbl, as well as tyrosine 1045 of EGFR, are required for receptor neddylation. A, CHO cells were transfected with expression vectors for Myc-Nedd8, and where indicated, c-Cbl, together with plasmids encoding EGFR proteins, either wild type (WT), a kinase-dead mutant (K721A), or the Cbl docking site mutant (Y1045F). Cells were treated with EGF and extracts were analyzed as described in the legend to Fig. 1A. Note the EGF-induced phosphorylation of a kinase-dead EGFR, presumably via ErbB-2. P-Tyr, phosphorytosine. B, CHO cells were transiently transfected with vectors encoding EGFR and Myc-tagged Nedd8, together with HA-tagged Cbl proteins. Analysis was performed with the indicated antibodies. C, left, domain structure of wild type c-Cbl and various mutants. Indicated are a domain related to Src homology 2 (TKB), the RING finger (RF), the proline-rich (PR) region, and the leucine zipper (LZ). Individually mutated residues are marked. C, right, CHO cells were transfected with vectors encoding EGFR and Myc-Nedd8, along with various c-Cbl expression constructs. Forty-eight hours post-transfection, cells were treated without or with EGF and cell extracts were analyzed with the antibodies indicated. D, Bosc cells were transiently transfected with FLAG-tagged expression vectors for Ubc12 or UbcH7 (2 μg each), together with HA-c-Cbl encoding plasmids, either wild type or 70Z, as indicated. Cell lysates were subjected to analyses 48 h post-transfection. E, EGFR immunoprecipitates (IP) from A431 cells were subjected to an in vitro Nedd8 conjugation reaction in the presence of rabbit reticulocyte lysate and 125I-labeled recombinant Nedd8 (15 ng). GST-Cbl (25 ng) and AG1478 (100 nM) were added as indicated. Free ubiquitin was added where indicated at 10- or 20-fold molar excess relative to Nedd8. Reaction mixtures were incubated for 1 h at 30 °C and subjected to autoradiography after electrophoresis.
Nedd8 Conjugation to EGFR

A Dominant-negative Mutant of Ubc12, as Well as Knockdown of Nedd8 Expression, Inhibits EGFR Down-regulation—Replacement of a cysteine residue conserved in the E2 family with a serine creates an effective dominant-negative mutant of Ubc12, which forms stable ester-bonded complexes with Nedd8 (33). Hence, we used Ubc12-C111S to sequester the endogenous Nedd8 of living cells. The results presented in Fig. 3A confirm that Ubc12-C111S covalently sequesters the endogenous Nedd8 of Bosc cells, as well as ectopic Myc-tagged Nedd8 molecules (bottom panel of Fig. 3A). As expected, Nedd8 sequestration inhibited receptor neddylation. Importantly, it also led to a reduction in ligand-induced degradation of EGFR and a significant enhancement of receptor expression (Fig. 3A). Along with other lines of evidence, these results establish a role for endogenous Nedd8 molecules in ligand-induced receptor degradation.

CHO cells expressing the dominant-negative form of Ubc12 (Ubc12-C111S) displayed retarded receptor down-regulation, but cells ectopically expressing an inert mutant (C111A) exhibited normal receptor behavior (Fig. 3B). Thus, depletion of endogenous Nedd8 suggests that neddylation is essential for EGFR endocytosis. To test this conclusion, we used RNA interference. Three pSuper vectors (27) containing 19-nucleotide long inserts derived from nedd8 were tested. Because anti-Nedd8 antibodies better detect the conjugated protein than the free molecule, we utilized the aforementioned ability of Ubc12-C111S to bind Nedd8. When expressed in CHO cells, Ubc12-C111S displayed a protein band that was recognized by antibodies to Nedd8 (Fig. 3C). Upon co-expression of Nedd8 fused to three Myc peptide tags we observed an additional band corresponding to an Ubc12-(Myc)3-Nedd8 complex. Utilizing this system, we identified one siRNA construct (construct C), which reproducibly reduced the level of Ubc12-Nedd8 (Fig. 3C). Introduction of this construct, along with c-Cbl and EGFR, into CHO cells verified the ability of the vector to eliminate conjugation of ectopic Nedd8 to EGFR, and also revealed complete blockade of EGF-induced receptor degradation (Fig. 3D). These results established the efficacy of the siRNA approach, and enabled us to examine the involvement of the endogenous Nedd8 in receptor down-regulation (Fig. 3E). Depletion of endogenous Nedd8 almost completely blocked the initial phase of receptor down-regulation, but the inhibitory effect gradually decreased. Whereas complete blockade of receptor endocytosis

FIGURE 3. A dominant-negative mutant of E2-Nedd8 (Ubc12), as well as knockdown of Nedd8 expression, inhibits EGFR down-regulation. A, Bosc cells were co-transfected with vectors expressing EGFR, c-Cbl, and Myc-Nedd8, along with increasing amounts of a plasmid encoding FLAG-tagged Ubc12-C111S. Forty-eight hours later, cells were treated with EGF and analyzed as indicated. Bands corresponding to Ubc12 are marked. Note the covalent conjugation of Ubc12-C111S to the endogenous Nedd8. B, receptor down-regulation assay: CHO cells were transiently transfected with an EGFR expression vector, along with the following plasmids: an empty vector (closed circles), or combinations of a c-Cbl plasmid with either a control plasmid (closed triangles) or vectors encoding Ubc12, either C111S (open triangles) or C111A (open squares). Forty-eight hours after transfection quadruplicate monolayers were treated with EGF at 37°C and receptor down-regulation analyzed. Average values ± S.D. (bars) are shown. The inset shows immunoblotting (IB) results obtained with EGF-treated (15 min) CHO cells, used in the down-regulation assay, and co-expressing c-Cbl and the indicated forms of Ubc12 (or an empty vector). C, CHO cells were transiently transfected with vectors encoding Ubc12-C111S and as indicated Myc-Nedd8, as well as pSuper-Nedd8 constructs (A, nucleotides 148–167 from the start site; B, 153–172; C, 162–181) or an empty pSuper. After 48 h, cell lysates were subjected to analyses with the antibodies indicated. Bands corresponding to covalent Ubc12-C111S-Nedd8 conjugates are marked. The arrow refers to a nonspecific band, just above a minor band corresponding to adducts of Ubc12 and endogenous Nedd8. D, CHO cells were transfected with plasmids encoding EGFR, c-Cbl, and Myc-Nedd8, along with the empty pSuper plasmid or pSuper-Nedd8 (construct C). Forty-eight hours after transfection, cells were incubated without or with EGF for 15 min, and analyzed as indicated. E, CHO cells were transfected with an EGFR plasmid, either alone (closed circles) or in combination with c-Cbl and control pSuper vectors (closed triangles), or c-Cbl and pSuper-Nedd8 (construct C, open triangles). Forty-eight hours post-transfection, quadruplicate monolayers were treated with EGF and analyzed in a down-regulation assay. IP, immunoprecipitating.
Receptor Down-regulation Is Defective in ts41 Cells, but the E1 Activating Enzyme for Nedd8 Can Rescue the Defect—The ts41 mutant was derived from mutagen-treated CHO cells (E36) and it leads to cell cycle defects (34). Ectopic APP-BP1 gene, which encodes a subunit of the E1 activating enzyme for Nedd8, rescues the defect of ts41 cells (35). As an independent test of the biological significance of NFRG neddylation, we analyzed ligand-induced degradation of EGFR in ts41 cells grown at the permissive (32 °C) or restrictive (40 °C) temperatures, in comparison to the parental E36 cells (Fig. 4A). Whereas at 32 °C the two cell lines displayed similar kinetics of ligand-induced receptor degradation, this process was relatively slow in ts41 cells grown at the restrictive temperature. In line with partial dependence of EGFR degradation on intact neddylation, when stably expressed in ts41 cells and tested at 40 °C, EGFR displayed retarded down-regulation (Fig. 4B).

To directly relate the defect of ts41 cells to the neddylation system, we repeated the down-regulation assay in cells transiently expressing the genetically defective component, APP-BP1. Due to ectopic expression of EGFR, which is known to saturate the endocytic pathway (30), the extent of receptor down-regulation was limited, especially at 32 °C (Fig. 4C). Nevertheless, the ectopically expressed E1 component of Nedd8 significantly enhanced receptor down-regulation. As expected, APP-BP1 elicited no effect on the rate of receptor down-regulation in E36 cells or in ts41 cells maintained at the permissive temperature (Fig. 4C). Because it was postulated that Cbl plays a critical role at the level of endosomal sorting, rather than at the level of internalization (23, 36), we analyzed EGFR internalization rates. As expected, the uptake of a radioactive EGF at 32 °C was slower than the rate of internalization at 40 °C. Moreover, ts41 and E36 cells displayed essentially identical rates (Fig. 4D), in line with intactness of the endocytic machinery of ts41 cells and a non-essential role for Cbl-mediated neddylation/ubiquitylation in the initial phase of endocytosis. In summary, the results obtained with ts41 cells confirm a role for the neddylation machinery in late endocytic sorting of EGFR.

Nedd8 Conjugation Enhances Ligand-induced Ubiquitylation of EGFR—We next examined the possibility that neddylated EGFR molecules serve as better targets for ubiquitylation. Sequestering endogenous Nedd8 molecules of CHO cells using an overexpressed Ubc12-C111S remarkably reduced the extent of EGF-induced receptor ubiquitylation, and also moderately stabilized EGFR (Fig. 5A). Another experimental approach entailed expressing ectopic Nedd8 at very low levels. Under these conditions receptor neddylation was barely detectable (Fig. 5B and data not shown), but ubiquitylation was clearly enhanced. Hence, both results support the notion that receptor neddylation enhances subsequent ubiquitylation. In line with this conclusion, the relatively effective knockdown of the endogenous Nedd8 of CHO cells using siRNA remarkably inhibited ligand-induced ubiquitylation and degradation of EGFR (Fig. 5C). Conceivably, by enhancing ubiquitylation, Nedd8 accelerates receptor down-regulation.

Neddylation and Ubiquitylation Sites Are Shared and Include the Kinase Domain of EGFR—The cytoplasmic domain of EGFR contains multiple lysines, including 22 residues confined to the kinase domain, whose activity is essential for Cbl recruitment. To address neddylation sites without affecting the catalytic activity of EGFR, we analyzed a truncated receptor, in which all lysine residues not included within the kinase domain were mutated to arginines. The mutant retained ligand-induced neddylation and degradation, as well as a high molecular weight smeary appearance (Fig. 6A), indicating that multiple lysines located within the kinase domain serve as primary or surrogate targets for neddylation. According to a recent proteomic analysis of EGFR, several lysine residues located within the kinase domain serve as ubiquitylation sites (9). Hence, it is conceivable that both Nedd8 and ubiquitin molecules co-exist on stimulated EGFRs. Indeed, co-expression of Myc-Nedd8 and HA-ubiquitin followed by immunoprecipitation with anti-Myc antibodies, under conditions that dissociate protein-protein interactions, detected EGFR molecules conjugated to both Nedd8 and ubiquitin (Fig. 6B).

To address the relationships between receptor neddylation and ubiquitylation, we co-expressed peptide-tagged Nedd8 and ubiquitin and analyzed EGF-treated, c-Cbl-expressing cells. The results presented in Fig. 6C show a biphasic pattern: upon graded overexpression of Nedd8 in EGF-stimulated CHO cells, ubiquitylation of EGFR gradually increased, in line with other observations (Fig. 5), and then significantly decreased. In contrast, a monotonic pattern of inhibited neddylation was observed upon graded overexpression of ubiquitin. This behavior is consistent with the possibility that neddylation promotes ubiquitylation of EGFR, but when present in excess, Nedd8 competes ubiquitin out of shared target lysines.

Nedd8 and Ubiquitin Distinctly Regulate the Endocytic Machinery—Evidence from yeast indicates that a ubiquitin ligase called Rsp5p ubiquitylates a component of the endocytic machinery.
machinery, prior to endocytosis of membrane proteins (37). Rsp5p and its mammalian orthologs, Nedd4 family HECT domain ligases, monoubiquitylate UIM-containing components of the endocytic machinery, including Eps15, Hgs/Hrs, and STAM (reviewed in Ref. 11). When ectopically expressed in CHO cells, all three proteins underwent ubiquitylation but no neddylation (Fig. 7A), suggesting a major difference between Cbl and Nedd4 families of E3 ligases. To further prove that UIM-containing proteins undergo no neddylation, we utilized the ability of isolated UIMs to target Nedd4-mediated ubiquitylation in intact cells (26). When fused to GST, the UIM of Hgs conferred extensive ubiquitylation, but no detectable neddylation (Fig. 7B), even when Nedd4 was overexpressed and ubiquitylation increased (Fig. 7C). In conclusion, unlike RING E3s, HECT ligases cannot function as dual specificity enzymes capable of utilizing both ubiquitin and Nedd8.

Along with an ability to recruit Nedd4 and other HECT ligases, the UIM specifically binds monoubiquitin with modest affinity (38). Hence, we examined the possibility that UIM<sup>UIMs</sup> will bind Nedd8. To this end we expressed in bacteria GST fusion proteins comprising either Nedd8 or ubiquitin, and tested their interactions with the endogenous Hgs/Hrs of HEK-293T cells (Fig. 7D). Unlike control GST, GST-ubiquitin bound a significant fraction of input Hgs, whereas GST-Nedd8 displayed lower but detectable interaction with Hgs. To ascertain that the UIM acts as the recognition site, we tested a mutant lacking the UIM. Although the shorter mutant reached expression levels comparable with the endogenous Hgs, it lost the ability to recognize both Nedd8 and ubiquitin (Fig. 7D).

These results imply that sorting is dictated by the ability of the UIM to bind ligand-activated cargoes through their appended Nedd8 and ubiquitin molecules. To test this scenario, we stimulated HeLa cells with EGF and examined binding of the stimulated or unstimulated EGFR with recombinant portions of Hgs. The data shown in Fig. 7E indicate that a portion of Hgs containing the UIM specifically binds ligand-activated receptors, and completely avoids the unmodified EGFR. In summary, the UIM is able to bind both Nedd8 and ubiquitin, although only the latter is targeted to substrates through the concerted action of HECT E3s and UIMs. Hence, by enhancing both neddylation and ubiquitylation of cargoes like EGFR, the Cbl machinery ensures robust desensitization of activated receptor tyrosine kinases.

**DISCUSSION**

Evidence from gene-targeted mice (39) and hamster cells defective in neddylation (34) indicate that Nedd8 and ubiquitin fulfill non-redundant functions. One clear difference is the existence of enzymatic systems that prevent diversion of Nedd8 into the ubiquitin pathway (40). Another difference relates to the two sets of proteins targeted by each modifier. The present study adds a transmembrane receptor tyrosine kinase to the list of neddylation substrates, which includes the cell cycle regulators Cullins (13, 41, 42) and p53 (22).

RING-containing ubiquitin ligases like c-Cbl act as scaffolds...
that assemble ubiquitin-loaded E2 molecules and their substrates. Here we demonstrated in living cells and in a cell-free system that Cbl also conjugates Nedd8 to ligand-activated EGFR molecules. Neddylation, as well as ubiquitylation of EGFR, depends on both the TKB domain and the RING finger of c-Cbl (Fig. 2), because the former binds to phosphorylated tyrosine 1045 of EGFR and the latter recruits an E2 molecule. The key for the duality of c-Cbl is the apparent non-discriminative nature of its interaction with E2 molecules loaded with either Nedd8 (Ubc12) or ubiquitin (UbcH7; Fig. 2D). Consistent with this explanation, the RING of c-Cbl contacts two loops of UbcH7, which are homologous to the corresponding loops of Ubc12 (43). The promiscuous nature of c-Cbl as an E3 ligase is analogous to the ability of UbcH8, an E2 enzyme, to conjugate both ubiquitin and the related protein ISG15 (44), but it stands in contrast to the ubiquitin-only specificity of HECT E3s (Fig. 7). Like c-Cbl, two other RING proteins, namely Roc1 (15, 17) and Mdm2 (22) act as dual specificity ligases. Hence, it is possible that additional RING fingers recruit Ubc12 and mediate neddylation. Another issue relates to lysine residues of neddylation substrates. Mdm2-mediated ubiquitylation of p53 modifies six carboxyl-terminal lysines, only three of which are neddylated by Mdm2 (22). Similarly, only one lysine of Cullin1 (residue 720 (45)) undergoes neddylation by Roc1. Our results suggest that lysine residues located in non-catalytic regions of EGFR are not essential, and may not serve as targets for this modification (Fig. 6A). In addition, based upon the appearance of neddylated bands of the receptor we assume that multiple lysines within the kinase domain of EGFR undergo neddylation. The multiplicity of neddylation sites, their localization to the kinase domain, as well as the abundance of appended Nedd8 molecules, are remarkably reminiscent of the reported pattern of EGFR ubiquitylation (9).

Three experimental tools proved useful in relating Nedd8 to endocytosis, namely siRNA of Nedd8 (Fig. 3E), a dominantly-negative mutant of Ubc12 (Fig. 3, A and B), and ts41 cells defec-
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**A**

**B**

**C**

**D**

**E**

FIGURE 7. The UIM of Hgs recognizes Nedd8 but mediates autoubiquitylation, not autoneddylation. A, CHO cells transiently expressing Myc-Nedd8 or Myc-ubiquitin, along with the indicated peptide-tagged UIM-containing proteins, were extracted and the extracts were analyzed either directly or after immunoprecipitation of the respective UIM-containing protein. B, CHO cells were transfected with the indicated GST fusion proteins (GST alone or GST-UIM<sup>99p</sup>) and a Myc-tagged modifier (Nedd8 or ubiquitin). Cell extracts were analyzed either directly or after pull down (PD) on glutathione beads. C, CHO cells were co-transfected with plasmids encoding GST-UIM<sup>99p</sup> and a Myc-tagged modifier (Nedd8 or ubiquitin), along with increasing concentrations of a Nedd4 plasmid. Analyses were performed as in B. D, extracts of CHO cells transfected with either a control plasmid or a vector encoding Hgs-UIM were subjected to immunoblotting with anti-Hgs antibodies, either directly (lanes labeled none) or after pull down with the indicated GST fusion proteins. Note the binding of the endogenous Hgs, and not the ectopic mutant, to ubiquitin and Nedd8. E, serum-starved HeLa cells were untreated or stimulated with EGF (10 min), and cleared whole cell extracts were analyzed directly (input), or incubated for 1 h at 4 °C with the indicated GST fusion proteins. Proteins bound to glutathione-agarose beads were resolved by electrophoresis and detected with an antibody to EGFR. The lower panel shows a stained gel with the respective GST fusion proteins.

tive in protein neddylation (Fig. 4). In contrast, siRNA of c-Cbl was unable to affect receptor neddylation (data not shown), probably due to the multiplicity of Cbl proteins. Consistent with a role in cargo sorting, ectopic Nedd8 accelerated ligand-induced degradation of EGFR (Fig. 1E), whereas depletion of endogenous Nedd8 using either siRNA or Ubc12-C111S, stabilized EGFR and decelerated the rate of receptor down-regulation. Two mechanisms seem to underlie sorting of Nedd8-modified EGFR molecules. First, the multiple Nedd8 moieties decorating EGFR likely serve as docking sites that avidly recruit UIMs of clathrin adaptors such as Epsin. In addition, Nedd8 exerts a stimulatory effect on receptor ubiquitylation, similar to the role it plays in Cullin-dependent ubiquitylation (reviewed in Ref. 21). Accordingly, enhanced expression of Nedd8 is accompanied by elevated ubiquitylation of EGFR (Fig. 5B), while limiting Nedd8 availability decreases receptor ubiquitylation (Fig. 5, A and C). In agreement, gradual overexpression of Nedd8 increased, then decreased, receptor ubiquitylation (Fig. 6C). In analogy to the role for Nedd8 in ubiquitylation mediated by Cullins (46), we attribute the enhancing effect of appended Nedd8 molecules to their charged surfaces, which may expose to ubiquitylation buried lysines of EGFR.

A multiprotein complex physically recruits cargoes to clathrin-coated regions of the plasma membrane (reviewed in Ref. 3). Two features are common to components of this machinery: they undergo monoubiquitylation by Nedd4 E3s, and their ubiquitin-binding regions (e.g. UIMs) recognize monoubiquitins of cargoes, as well as other components of the complex. The data presented in Fig. 7 indicate that Nedd8, like ubiquitin, binds to UIMs of the endocytic machinery, but none of three UIM-containing proteins we tested undergoes neddylation. Thus, the UIM emerges as a domain that binds both ubiquitin and Nedd8, but by means of recruiting Nedd4 it targets autoubiquitylation and not autoneddylation (Fig. 7). The combined action of the UIM as an ubiquitin binder and as a domain that targets autoubiquitylation has been attributed to its ability to bind a thioester Nedd4-ubiquitin intermediate (11). We assume that the analogous Nedd4-Nedd8 complex does not
exist because the respective E2, Ubc12, cannot load Nedd8 onto Nedd4. Hence, this model explains why scaffold E3s like c-Cbl carry out both ubiquitylation and neddylation, whereas catalytic E3s like Nedd4 can perform ubiquitylation and not neddylation.

In summary, our data add an essential step to the chain of events that sort receptor tyrosine kinases for endocytosis. In the case of EGFR, the process initiates with ligand-induced receptor phosphorylation, which recruits Cbl molecules pre-complexed to Ubc12. Subsequently, Ubc12 downloads monomeric Nedd8 onto several lysines located within the kinase domain. This promotes enhanced recruitment of the alternative Cbl-UbcH7 complex, which conjugates ubiquitin to additional lysine receptors. In the next step the appended Nedd8 and ubiquitin moieties dock UIM-containing adaptors like Epsin and Hgs, thereby linking the modified receptor to clathrin-coated regions of the plasma membrane and endosomes, respectively. Along with refined resolution of the sorting process, this model leaves open several issues, such as the role played by de-neddylation enzymes and possible kinase inactivation through neddylation/ubiquitylation.

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