Cbl proteins have RING finger-dependent ubiquitin ligase (E3) activity that is essential for down-regulation of tyrosine kinases. Here we establish that two WW domain HECT E3s, Ned4 and Itch, bind Cbl proteins and target them for proteasomal degradation. This is dependent on the E3 activity of the HECT E3s but not on that of Cbl. Consistent with these observations, in cells expressing the epidermal growth factor receptor, Ned4 reverses Cbl-b effects on receptor down-regulation, ubiquitylation, and proximal events in signaling. Cbl-b also targets active Src for degradation in cells, and Ned4 similarly reverses Cbl-mediated Src degradation. These findings establish that RING finger E3s can be substrates, not only for autoubiquitylation but also for ubiquitylation by HECT E3s and suggest an additional level of regulation for Cbl substrates including protein-tyrosine kinases.

Specificity in ubiquitylation is conferred by ubiquitin (Ub) protein ligases (E3s). These mediate the transfer of Ub from Ub-conjugating enzymes (E2s) to substrates or to a Ub that is already linked to substrate. There are two major classes of E3s: those that are homologous to the E6-AP carboxyl terminus (HECT) and RING finger and RING finger-like E3s (1–4). As part of the ubiquitylation process, HECT E3s form thiol-ester (HECT) and RING finger and RING finger-like E3s (1–4). As part of the ubiquitylation process, HECT E3s form thiol-ester catalytic intermediates with Ub. For RING finger and related E3s, there is no evidence for such intermediates (1, 2).

A large number of yeast and metazoan plasma membrane receptors and transporters are substrates for ubiquitylation, which generally leads to their degradation in the yeast vacuole or metazoan lysosome. In Saccharomyces cerevisiae, the HECT domain E3, Rsp5p, is implicated in the ubiquitylation of many membrane proteins (5, 6). This E3 also contains multiple WW domains in its amino-terminal half that are responsible for substrate binding. WW domains interact with a variety of proline-based motifs, and certain WW domains also recognize proline-containing phosphoserine/phosphothreonine sequences (7, 8). There are several metazoan Rsp5p homologs. The most closely related to Rsp5 and also the best characterized is Ned4 (also known as Ned4-1). A highly homologous protein, Ned4-2, which is the product of a different gene, has more recently been identified (9). Ned4 and Ned4-2 are implicated in the ubiquitylation and lysosomal targeting of the epithelial sodium channel (ENaC), as is the related family member WWP2 (9–11). Closely related to these are WWP1 and Itch, also known as Alp4, which has been implicated in the Notch signaling pathway (1, 2, 12, 13). Other WW domain HECT E3s that share significant homology with these include SMURF-1 and -2, which are involved in degradation of SMAD proteins (1, 2). Most WW-HECT E3s also have a calcium-dependent lipid-binding (C2) domain at the extreme amino terminus that mediates plasma membrane association (14). In addition to ENaC, Ned4 ubiquitylates other substrates including some that reside at, or transit through, the plasma membrane such as the Ras guanine nucleotide exchange factor (15). Ned4-mediated ubiquitylation is also implicated in viral budding from the plasma membrane (16, 17). EPS15 and other Ub-interacting motif (UIM) proteins involved in endocytosis and lysosomal trafficking of receptors are also Ned4 substrates (18, 19). For another class of membrane proteins, the receptor tyrosine kinases (RTKs), the Cbl family of RING finger E3s are generally implicated in down-regulation with evidence that they mediate RTK ubiquitylation as part of this process (20–22). RTKs for which Cbl proteins have been most clearly implicated as cognate E3s include the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (23–26). Mammalian Cbl proteins include c-Cbl, Cbl-b, and Cbl-c (also known as Cbl-3). c-Cbl has also been shown to play a role in the degradation of nonreceptor tyrosine kinases including Src, Fyn, and Syk (20–22). Mutant forms of c-Cbl that function as “dominant negatives” have been shown to have transforming activity, presumably as a consequence of decreased turnover and thus increased activity of tyrosine kinases (20, 21). Recruitment of Cbl family members to activated RTKs occurs through interactions involving the highly conserved amino-terminal phosphotyrosine binding domains of the Cbl proteins. c-Cbl and Cbl-b are closely related proteins with extensive proline-rich regions that can bind a variety of Src homology 3-containing proteins implicated in signaling and receptor down-regulation (20, 21, 27, 28). Both also have C-terminal Ub-associated (UBA) domains. Cbl-c is the most recently described member of the family (29, 30), and unlike c-Cbl and Cbl-b (22, 31–34), no specific developmental function has been ascribed to it. Cbl-c has a more limited proline-rich region and contains neither a
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UBA domain nor the C-terminal tyrosine phosphorylation sites found on c-Cbl and Cbl-b (20–22).

Since Nedd4 and Cbl both ubiquitylate plasma membrane proteins and Cbl proteins all include potential sites of interaction with WW domains, we asked whether they might physically and functionally interact. We report here that Nedd4, as well as Itch, associates with all three mammalian Cbl proteins and regulates their protein levels by ubiquitylating them for ubiquitin-dependent proteasomal degradation. Consequently, Nedd4 inhibits Cbl-b-mediated ubiquitylation and down-regulation of the EGFR and reverses effects on proximal events in signaling through this receptor. Similarly, Nedd4 reverses Cbl-mediated down-regulation of activated Src. These data demonstrate negative regulation of RING finger E3s by HECT family E3s and point to the potential for an additional level of complexity in the regulation of protein-tyrosine kinases.

EXPERIMENTAL PROCEDURES

Plasmids—HA epitope-tagged Cbl-b, Cbl-b(C3407A) and Cbl-b N1/3 have been described (35). Plasmid encoding the Cbl-b proline-rich (PR) domain (aa 482–721) was generated by digestion of wild type Cbl-b with BamHI and EcoRI and ligation into pEC20N from Rho/G to EcoRI to generate pEC20N-Cbl-b PR. Plasmid encoding the Cbl-b RING finger (aa 327–483) was generated by digestion of Cbl-b C-terminal 2/3 (35) with BamHI and XhoI and religation. Plasmid encoding Cbl-b without epitope tag was generated by site-specific mutagenesis by introduction of a stop codon before the HA tag: pcDNA-HA-Nedd4 was generated by subcloning of pc-HA-Nedd4 (36) into pCDNA3.1 (+) using NcoI and EcoRI. Plasmid encoding Nedd4 in which the active site cysteine was mutated to serine (pc-HA-Nedd4(C3407A)) was generated by site-directed mutagenesis (QuikChange kit; Stratagene, La Jolla CA). N-terminal Myc-tagged mouse Itch was generated by subcloning of full-length Itch (12) into pCI-neo using Nhel (5') and NotI (3') sites; the Myc tag was generated by PCR. The Cys to Gly mutation of the active site Cys of Itch was carried out by site-specific mutagenesis. Glutathione S-transferase (GST)-Nedd4, GST-Nedd4-N, GST-Nedd4-C (36); GST-ITch (37); EGFR (38); S src (39); Myc-E6-AP (36); HA-Cbl-c (29); and HA-Ub (40) have been described. pEGFP-C2 encoding GFP was from Invitrogen.

Antibodies and Reagents—Anti-Cbl-b (H121; H145), anti-GFP (FL), anti-c-Cbl (C-15), anti-Src (N16), anti-EGFR (10D5), anti-Myc 9E10 (SC-40), and anti-MAPK (C-14) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-EGFR (Ab-3) was from Oncogene Science (Beverly, MA); and anti-HA epitope (12CA5) was from culture supernatant. Anti-Ub has been described (41). Anti-Nedd4 was raised in rabbit by immunization with a GST fusion of mouse Nedd4 (36). Unless otherwise noted, reagents were used at the following concentrations in cell experiments: lactacystin, 20 μM (Calbiochem); cycloheximide, 100 μM; ammonium chloride, 20 mM; recombinant human EGFR, 100 ng/ml (Pepro Tech Inc., Rocky Hill, NJ).

Cell Experiments—The Jurkat human T-cell leukemia cell line (41) and 293T human embryonic kidney cells expressing SV40 large T antigen (37) were maintained as described. Transfections in 293T cells were with calcium phosphate (5 Prime – 3 Prime, Inc., Boulder, CO), Superfect (Qiagen, Valencia, CA), or LipofectAMINE 2000 (Invitrogen). Transfections into HeLa cells were with LipofectAMINE Plus (Invitrogen). For experiments involving Cbl-c, cells were plated at 0.25 × 10^6 in 100-mm dishes. For all other experiments, 0.5 × 10^6 cells were plated in 100-mm dishes. Transfections were carried out the following day. Cbl constructs expressed at a considerably better level than Nedd4; therefore, in general, 0.5–3.0 μg of Cbl encoding plasmid and 5–12 μg of Nedd4 plasmids were used for transfections. In all transfection experiments, the total amount of plasmid was equalized among samples using the appropriate expression vector without insert (V) so that equal amounts of promoter were transfected and expressed in each transfection. Cells were washed at 4 °C in PBS containing 0.5 mg/ml sodium orthovanadate and, unless otherwise indicated, lysed at 4 °C in lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% β-ocetyl glucoside (Sigma), 10% glycerol, 10 mM iodoacetamide, 2 mM orthovanadate, and protease inhibitors as described (41). Lysates were cleared at 14,000 rpm for 15 min at 4 °C. Cell lysates were either first analyzed by immunoprecipitation or directly resolved by SDS-polyacrylamide gel electrophoresis followed by transfer to polyvinylidene difluoride membrane. Immunoprecipitations were carried out using antibodies and protein A-agarose. The wash buffer used in immunoprecipitation differed from the lysis buffer in being 0.1% in both Triton X-100 and β-ocetyl glucoside. Proteins were detected by immunoblotting using standard methods, either ECL (Amersham Biosciences) or [125I]-protein A after incubation with primary antibodies. [125I]-Quantification was by Storm PhosphorImager and ImageQuant software (Amersham Biosciences). Proteasome and lysosomal inhibitors were added to cells for 8 h beginning 36 h after transfection. For experiments involving EGFR, 293T cells (70% confluence) were incubated overnight in serum-free medium (reduced from 10%). To evaluate Cbl-b ubiquitylation, cells were lysed in buffer containing 1% SDS to disrupt protein-protein interactions. After clearing of lysates, SDS was diluted 10-fold in lysis buffer. For Cbl-b ubiquitylation, HA-Ub was co-expressed to increase signal, but immunoblotting was with polyclonal anti-Ub.

In Vitro Binding and Ubiquitylation—GST fusion proteins expressed in Escherichia coli were prepared, stained using Coomasie Blue, and quantified by comparison with bovine serum albumin standards. Proteins were bound to glutathione-Sepharose as described (42). [35S]Methionine-labeled proteins were generated in rabbit reticulocyte lysate (37). For binding experiments, in vitro translation products were incubated with bead-bound GST fusion proteins for 2 h at 4 °C prior to washing in lysis buffer. In vitro ubiquitylation was carried out using glutathione-Sepharose-bound fusion proteins and added translation products as described (43) and was modified to include 100 μM MG132 (Biomol, Plymouth Meeting, PA) and 1 μM leupeptin (Boston Biochemicals, Cambridge, MA).

RESULTS

Nedd4 and Cbl-b Bind in Vitro—To ascertain whether Nedd4 and Cbl-b interact, GST fusion proteins of murine Nedd4 (36) were evaluated for binding to in vitro translated [35S]Methionine-labeled Cbl-b (Fig. 1B, upper panel; see Fig. 1A for schematics of Cbl-b and Nedd4). Significant binding of Cbl-b to Nedd4 (GST-Nedd4; aa 52–777) was observed. The N-terminal portion of Nedd4 (GST-Nedd4-N; aa 52–422), which contains the three WW domains but lacks the HECT domain, showed no bound Cbl-b. Under conditions where there was a substantial amount of Nedd4 bound to the C-terminal HECT domain (GST-Nedd4-C; aa 423–777), the domains of Cbl-b required for Nedd4 binding were next mapped (Fig. 1C). Nedd4 and Nedd4-N but not Nedd4-C demonstrated substantial binding to the proline-rich region of Cbl-b (Cbl-b PR) and to the nonoverlapping N-terminal third of Cbl-b (Cbl-b N1/3), which includes the phosphotyrosine binding domain as well as several noncanonical WW domain binding sites and a potential site of serine phosphorylation-dependent WW domain binding. In contrast, the RING finger and surrounding regions of Cbl-b did not bind Nedd4. Thus, Cbl-b and Nedd4 associate in a manner that does not require either protein’s Ub ligase domain.

Nedd4 and Cbl-b Associate in Cells—To evaluate whether Nedd4 and Cbl-b associate in cells, lysates from the Jurkat T-cell leukemia cell line, which expresses easily detectable levels of both Nedd4 and Cbl-b, were immunoprecipitated with either a polyclonal antibody raised against Nedd4 or anti-Cbl-b, followed by immunoblotting with antibodies directed against one or the other (Fig. 2A). Substantial specific immunoprecipitation of Nedd4 using anti-Cbl-b was detected (lane 1), as was immunoprecipitation of Cbl-b with anti-Nedd4 (lane 3). Both Cbl-b and Nedd4 associate with lipid rafts (5, 44, 45). Co-immunoprecipitation was not affected by the presence or absence of β-ocetylglucoside, which disrupts lipid rafts.

The co-immunoprecipitation of Cbl-b and Nedd4 suggests that a significant percentage of these molecules associate with each other in cells. If this is the case, preclearing of cell lysates with anti-Nedd4 would deplete a substantial percentage of Cbl-b. To evaluate this, lysates were first subject to immunoprecipitation with anti-Cbl-b followed by immunoblotting with antibodies directed against one or the other (Fig. 2A). Substantial specific immunoprecipitation of Nedd4 using anti-Cbl-b was detected (lane 1), as was immunoprecipitation of Cbl-b with anti-Nedd4 (lane 3). Both Cbl-b and Nedd4 associate with lipid rafts (5, 44, 45). Co-immunoprecipitation was not affected by the presence or absence of β-ocetylglucoside, which disrupts lipid rafts.

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Nedd4 and Cbl-b was also observed in 293T human embryonic kidney cells (293T cells) when Nedd4 and Cbl-b were co-transfected, since there is significant specific immunoprecipitation of Nedd4 with anti-Cbl-b (Fig. 2C, lane 4, middle panel). Thus, Cbl-b and Nedd4 demonstrate a strong physical association both in vitro and in cells.

Nedd4 Down-regulates Cbl Protein Levels—In transfected 293T cells, we noted that Cbl-b levels decreased when Nedd4 was co-expressed (Fig. 2C, upper panel, compare lanes 2 and 4). In contrast, Nedd4 levels were unaffected by co-expression of Cbl-b (Fig. 2C, lower panel, compare lanes 3 and 4). This suggested that Nedd4 might mediate loss of Cbl-b. As shown in Fig. 3A, Cbl-b levels did not decrease when co-expressed with Nedd4 in which the catalytic cysteine was mutated (Nedd4C744S). In contrast, expression of wild type Nedd4 at a comparable level markedly decreased Cbl-b. Thus, the loss of Cbl-b that we observe requires the Ub ligase activity of Nedd4.

The most likely explanation for our findings is that Cbl-b is being targeted for degradation by Nedd4. However, to exclude the possibility that the effect observed was occurring prior to protein synthesis, we measured the rate of loss of Cbl-b in the absence or presence of Nedd4, using cycloheximide to inhibit...
new protein synthesis (Fig. 3B). As is evident (Fig. 3B), in addition to decreasing the level of Cbl-b at the beginning of the cycloheximide treatment, expression of Ned4 markedly accelerated the rate of Cbl loss (t > 20 h without Ned4, ~5 h with Ned4). Levels of co-expressed c-Cbl as well as the smaller Cbl-c protein (data not shown and Fig. 3C) were similarly affected by Ned4, and consistent with this, both of these proteins also bound Ned4 (data not shown). Thus, Ned4 functionally and physically interacts with all three mammalian Cbl proteins.

To determine whether the Ned4-mediated loss of transfected Cbl extends to endogenous Cbl proteins, we examined endogenous Cbl levels in HeLa cells transfected with Ned4. Under conditions where >70% transfection efficiency was achieved, Ned4, but not catalytically inactive Ned4C744S, resulted in a striking loss of endogenous Cbl-b and c-Cbl (Fig. 3D, middle panels). Similar results were obtained in 293T cells for endogenous c-Cbl (data not shown), which is the only Cbl family member readily detectable in these cells.

To evaluate whether the Ned4-mediated loss of Cbl-b requires the E3 activity of Cbl-b, wild-type Cbl-b and RING mutant Cbl-b (Cbl-bC373A), which is inactive as an E3, were compared. As is evident (Fig. 4A), Ned4 enhanced the loss of RING finger mutant Cbl-b (Cbl-bC373A) as well as wild type Cbl-b. This establishes that the E3 activity of Cbl-b is not required for Ned4 to target it for degradation.

Ned4 Ubiquitylates Cbl-b and Targets It for Proteasomal Degradation—To determine whether Ned4-mediated loss of Cbl-b involves the Ub-proteasome pathway, we first assessed whether Ned4 ubiquitylates Cbl-b. To preclude autoubiquitylation of Cbl-b, cells were transfected with RING mutant Cbl-b (Cbl-bC373A), with or without co-transfection of Ned4. After treatment with lactacystin to inhibit proteasome function and lysis of cells in 1% SDS to disrupt the association between Cbl-b and Ned4, Cbl-b and Ned4 immunoprecipitates were evaluated for ubiquitylation by immunoblotting with anti-Ub (Fig. 4B). Expression of Ned4 resulted in a marked increase in ubiquitylated species immunoprecipitated with anti-Cbl-b (Fig. 4B, left upper panel). Ned4 was not detected on reprobing of blots (data not shown), indicating that the SDS did disrupt protein-protein interactions as expected and that the ubiquitylation observed does not represent co-associated Ned4 (data not shown). As further confirmation that the ubiquitylation seen with Ned4 co-expression was of Cbl-b and not Ned4, anti-Nedd4 immunoprecipitates from an equal amount of the same lysates were evaluated for ubiquitylation. No increase in ubiquitylation was observed with Ned4 expression (right upper panel). Similar results were obtained with co-expression of wild type Cbl-b and Ned4 (data not shown). These findings strongly suggest that Ned4 is mediating Cbl-b ubiquitylation in vivo.

To further evaluate the capacity of Ned4 to directly ubiquitylate Cbl-b, we carried out in vitro ubiquitylation reactions. In vitro translated Cbl-bC373A was incubated with GST, GST-Ned4, or the catalytically inactive GST-Ned4-N. A substantial decrease in the Cbl-b band and a marked increase in higher molecular weight forms, indicative of ubiquitylation, was seen only with GST-Ned4 and only when E1 and E2 were added (Fig. 4C, lane 6). Thus, Ned4 mediates Cbl-b ubiquitylation both in vivo and in vitro.

We have previously shown that Cbl-b is degraded in response to EGFR stimulation apparently as part of the coordinated degradation of EGFR signaling complexes (35). This activation-dependent degradation requires both proteasome and lysosome function. To determine whether Cbl-b that is ubiquitylated by Ned4 is proteasomally degraded versus lysosomally degraded or requires both activities, cells were treated with either the proteasome inhibitor lactacystin or with ammonium chloride, which inhibits lysosome function. Only the treatment with lactacystin prevented Ned4-mediated loss of Cbl-bC373A (Fig. 4D, upper panel, compare lanes 4–6). This was also found to be the case with wild type Cbl-b (data not shown; see also Fig. 4E, upper panel, lanes 4–6). Ned4-mediated loss of c-Cbl was also found to be proteasome-dependent (data not shown). To assure
that the ammonium chloride was functioning as expected in these experiments, EGF-induced loss of Cbl-b and EGFR was examined in parallel with Nedd4-mediated Cbl-b degradation (Fig. 4E). As expected, both ammonium chloride and lactacystin inhibited degradation of Cbl-b and EGFR in response to EGF (Fig. 4E, upper and middle panels, compare lanes 1 and 7–9). In contrast, only lactacystin inhibited the degradation of Cbl-b mediated by Nedd4 (Fig. 4E, upper panel, compare lanes 1 and 4–6). Thus, whereas the EGF-induced loss of Cbl-b is RING finger-dependent and requires both lysosome and proteasome activity (35), the targeting of Cbl-b for degradation by Nedd4 does not require EGFR activation, is RING finger-inde-
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Fig. 5. Cbl loss mediated by Itch but not E6-AP. A–C, cells were transfected with plasmid encoding the indicated proteins, and lysates were immunoblotted as indicated. In B and C, V indicates cells transfected only with plasmid without insert (vector). The middle panels in A and C were immunoblotted with both anti-HA to detect Nedd4 and with anti-Myc to detect Itch. Both Itch and E6-AP have N-terminal Myc tags. In C, the asterisk in the upper panel indicates a nonspecific band. IB, immunoblot; IP, immunoprecipitation.

Itch but Not E6-AP Targets Cbl Proteins for Degradation—To determine whether other WW domain-containing HECT E3s also interact with Cbl-b, we evaluated Itch and compared it with E6-AP, which includes a HECT domain but lacks WW domains in its amino-terminal half (36, 46). Consistent with the similarity between Nedd4 and Itch, GST-Itch but not GST-E6-AP bound in vitro translated Cbl-b (data not shown). Additionally, co-expression of Itch also resulted in loss of Cbl-b (Fig. 5A). This effect required the catalytic activity of Itch and could be reversed by treatment of cells with lactacystin (data not shown). In contrast, E6-AP failed to decrease Cbl-b, despite the fact that in the experiment shown E6-AP expression was substantially greater than Itch (Fig. 5B).

A recent study has described the in vivo physical interaction between Cbl-c and Itch (47). We have similarly observed that GST-Itch binds in vitro translated Cbl-c (data not shown). Moreover, as is shown by the representative experiment in Fig. 5C, we demonstrate that, in addition to their physical interaction, wild type Itch, but not Itch in which the active site cysteine has been mutated, mediated the loss of co-expressed Cbl-c. Thus, multiple WW domain HECT E3s target all three Cbl family proteins for degradation.

Nedd4 Blocks Cbl-mediated Degradation of Receptor and Nonreceptor Protein-tyrosine Kinases—The Nedd4-mediated decrease in Cbl levels would be expected to reverse effects of Cbl-b on the ubiquitylation and down-regulation of Cbl substrates. The best characterized of these is the EGFR, ubiquitylation and degradation of which is enhanced by co-expression of Cbl family proteins (24–26, 48). Accordingly, Cbl-b enhances ubiquitylation of the EGFR, and Nedd4 blocks this effect (Fig. 6A, upper panel, lanes 1–6). Expression of Nedd4 by itself does not result in any discernible EGFR ubiquitylation in response to ligand (Fig. 6A, lanes 7 and 8). Consistent with the abrogation of Cbl-dependent ubiquitylation of EGFR in response to ligand, Nedd4 also reverses the ligand-mediated EGFR down-regulation seen with Cbl-b (Fig. 6A, second panel from top, lanes 1–6; see also Fig. 6B, upper panel, and C, middle panel). Expression of Cbl proteins also frequently results in a decrease in basal levels of EGFR presumably due to enhanced constitutive degradation (49, 50). We observe this as well (Fig. 6B, upper panel, and 6C, middle panel; see also Fig. 4E) and, as might be expected, Nedd4 also reverses this effect and can result in an increase in EGFR levels above that seen even without expression of exogenous Cbl. This increase is consistent with targeting of endogenous Cbl proteins for degradation. Importantly, when catalytically inactive Nedd4 is co-expressed with Cbl-b, both the basal levels of EGFR and receptor down-regulation in response to EGF resembles that seen with Cbl-b alone (Fig. 6B, upper panel, compare lanes 3 and 4 with lanes 9 and 10). This effectively eliminates the possibility that the effects on EGFR observed with Nedd4 are simply due to overexpression of the WW or C2 domains. As previously described (35), Cbl is itself targeted for degradation in response to EGFR ligation. We have previously reported that this loss is best seen when Cbl levels are relatively low and EGFR levels are relatively high. This is in accord with the idea that only the pool of Cbls that become recruited to activated receptors are degraded (35). Consistent with this, the ability to detect a demonstrable decrease in total Cbl in response to EGF is greater when Cbl levels start out lower as a consequence of Nedd4 expression, although the absolute amount of Cbl lost is not increased (see Fig. 6, A–C, and accompanying legend). As might be expected given the effects on EGFR levels, Nedd4 also blocked Cbl-b-mediated attenuation of early downstream events in receptor signaling as assessed by MAPK activation (Fig. 6C, upper panel, compare lanes 2, 4, and 6). In accord with the lack of effect on EGFR levels, Nedd4 did not by itself affect EGFR-mediated MAPK activation (data not shown). Thus, all of the findings observed for the EGFR upon Nedd4 expression are consistent with reversal of Cbl effects on basal EGFR levels and EGFR down-regulation.

The factors that regulate EGFR signaling and turnover are
complex. In particular, there is evidence to suggest that Nedd4 also plays roles in the ubiquitylation of two UIM proteins, Eps15 and Hgs1, that are implicated in EGFR down-regulation (18, 19). Therefore, Nedd4 could potentially influence EGFR turnover at multiple steps. For this reason, we evaluated the role of Nedd4 in the degradation of another Cbl substrate. c-Cbl has been shown to ubiquitylate Src in vitro with evidence to suggest a role in its degradation (51). Consistent with a role for Cbl proteins in Src degradation, we observed a decrease in levels of constitutively active Src when Cbl-b was co-expressed (Fig. 6D, upper panel, lanes 2 and 3). This loss of active Src is proteasome-dependent and requires the RING finger of Cbl-b (data not shown). Cbl-b-mediated Src degradation was reduced by co-expression of Nedd4 (lane 4), and Nedd4 by itself did not affect Src levels (lane 5). Together, the data presented in Fig. 6 demonstrate that through the targeting of Cbl family proteins for degradation, Nedd4 inhibits Cbl-dependent down-regulation of both receptor and nonreceptor protein tyrosine kinases.

**DISCUSSION**

In this study, we establish that all three mammalian members of the Cbl family of RING finger E3s (c-Cbl, Cbl-b, and Cbl-c, also known as Cbl-3) interact with Nedd4 and Itch, which are members of the WW domain subfamily of HECT E3s. These results are consistent with the recent report of binding of Itch and Cbl-c (47). We demonstrate that, as a consequence of these interactions, Cbl proteins can be ubiquitylated by Nedd4 and targeted for proteasomal degradation. This reverses well characterized Cbl-b effects on EGFR (35) and also reverses Cbl-b down-regulation of activated Src (Fig. 6D).

For Cbl-b, two nonoverlapping domains, amino and carboxyl to the RING finger, are implicated in binding the WW domain-
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containing region of Nedd4. Unlike RING finger dimers such as BRCA1/BARD1 (52) and Mdm2/Mdmx (53), the interaction between Nedd4 and Cbl is independent of their Ub ligase domains. The amino-terminal C2 domain of Nedd4, which mediates interactions with lipid rafts, is similarly dispensable. Thus, whereas Nedd4 and Cbl can both be found in rafts (5, 44, 45), neither the C2 domain of Nedd4 nor rafts are required for binding. In addition to Nedd4 and Itch, the WW domain subfamily of HECT E3s encompasses other closely related and widely expressed proteins such as Nedd4-2, WWP1, and WWP2. Among these, WWP1 and WWP2 appear to be more closely related to Itch, whereas Nedd4-2 shares greater homology with Nedd4. Based on this, it is very likely that some or all of these also interact with Cbl proteins. Functional overlap among WW domain HECT E3 family members has a precedent; Nedd4, Nedd-2, and WWP2 all interact with ENaC (9–11).

Binding of these E3s results in ubiquitylation of Cbl proteins in cells and in vitro and the targeting of all three mammalian Cbls for proteasomal degradation. These observations represent the first demonstration of targeting of one class of Ub ligase for degradation by another. A notable aspect to the interaction between Nedd4 and Cbl-b is the striking asymmetry of the relationship between these E3s; Nedd4 is not noticeably destabilized by Cbl, even when the levels of Nedd4 are substantially less than Cbl (as assessed using a common epitope tag). Whereas the molecular basis for this remains to be determined, it underscores the remarkable degree of specificity in the regulation of ubiquitylation and proteasomal targeting in vivo. There are hundreds of RING fingers and over 30 HECT domains encoded in mammalian genomes. It now becomes of great interest to determine to what extent the physical and functional interactions demonstrated herein are recapitulated with other E3 pairs.

The capacity of Nedd4 to target both transfected and endogenous Cbls for degradation raises the question of why endogenous Cbls are detectable in cells that express both families of proteins. Other factors must play roles in modulating the activity and availability of endogenous Nedd4 and its relatives. One obvious factor is phosphorylation. Based on the bacterial expression of proteins used for binding and treatment of samples with phosphatases, there is no evidence to suggest a possible role for phosphorylation in mediating Nedd4-Cbl interactions. However, phosphorylation playing a negative regulatory role in vivo, as has been suggested for Nedd4-2 and ENaC, remains a possibility (54). Other factors that could modulate activity include compartmental segregation of the endogenous proteins or deubiquitylating enzymes that reverse ubiquitylation. There are a number of examples in the Ub literature where third party proteins affect E3 activity toward substrates. For example, ARF binds Mdm2 and inhibits p53 ubiquitylation without directly affecting p53-Mdm2 binding (55). Of particular relevance is the recent identification of a Nedd4-interacting protein that blocks the effect of Nedd4 on ENaC (56). Other Nedd4 binding partners have been identified that are not targeted for degradation and reside in the nucleus (57). These could sequester Nedd4 family members from non-nuclear substrates such as Cbls and ENaC. Dissecting out the factors involved in in vivo regulation of Cbl degradation will clearly require further study.

We previously reported that Cbl-b is itself ubiquitylated and targeted for degradation by EGFR activation (35). This ligand-dependent degradation is distinguished from what we report herein in several important ways. First, EGF-stimulated ubiquitylation and degradation of Cbl-b requires an intact RING finger, whereas that mediated by WW HECT E3s does not. Second, degradation of Cbl in response to ligand occurs coordinately with the degradation of EGFR and, as with EGFR loss, is sensitive to both proteasome and lysosome inhibitors. In contrast, WW HECT E3-mediated Cbl degradation requires only proteasome activity. Finally, the ability to easily discern loss of Cbl in response to EGFR activation becomes manifest when EGFR levels are high and Cbl levels are relatively low. This is in accord with the idea that only Cbls that become EGFR-associated are targeted. In contrast, Nedd4-dependent Cbl loss is readily apparent even when Cbl levels are quite high relative to Nedd4 (see Fig. 6A for an example). A question that arises is whether Nedd4 and related proteins also play roles in EGF-stimulated ubiquitylation of EGFR and Cbl. Such a role cannot be excluded simply on the basis of the fate of the ubiquitylated proteins (i.e. lysosomal versus proteasomal degradation). However, if Nedd4 is involved in EGF-stimulated ubiquitylation of the EGFR or of Cbl, an important mechanistic distinction from what we describe in this study would be a clear requirement for an intact Cbl RING finger (35).

It is now evident that in addition to members of the Cbl family, multiple other classes of proteins play roles in the ubiquitylation and down-regulation of growth factor receptors. Two UIM-containing proteins implicated in EGFR regulation, Eps15 and Hgs1, are potential Nedd4 substrates. Monoubiquitylation of Eps15 by Nedd4 is postulated to increase EGFR loss (18), whereas degradation of Hgs1 due to Nedd4-mediated ubiquitylation is suggested to have the opposite effect, increasing basal EGFR levels (19). However, for neither Eps15 nor Hgs1 has the role of Nedd4-mediated ubiquitylation in modulating EGFR levels been directly addressed. Our study provides evidence that both Nedd4 and Itch target Cbl family members for degradation. The net effect of Nedd4 on EGFR is consistent with Nedd4 reversing well established effects of Cbl-b and c-Cbl on EGFR. However, in light of the UIM data, we cannot rule out the possibility that other effects of Nedd4 contribute to what we observe. In particular, the increase in basal EGFR levels with Nedd4 would also be consistent with the predicted effect of down-regulating Hgs1 by Nedd4. It is also clear that extrapolating from the EGFR to all RTKs is not necessarily valid. In this regard, a recent report provides evidence that ubiquitylation of the insulin-like growth factor receptor-1, for which there are no data implicating Cbls, may occur as a consequence of interactions involving the Nedd4 C2 domain and insulin-like growth factor receptor-1-associated Grb10 (58).

All of these observations in metazoan systems, derived from in vitro analyses and transfection studies, raise the question of the in vivo significance of the various ubiquitylation events and interactions in RTK regulation. Dissecting out the relative importance of at least five WW domain HECT E3s, multiple UIM proteins, and three different Cbls will require a capacity for combinatorial modulation of multiple members of these families. However, even as such modulation becomes feasible in cell culture, it will remain to be determined to what extent redundancies that might be observed accurately reflect the situation in various tissues in situ. For example, despite a level of redundancy in cultured cells and in embryonic development, mice negative for Cbl-b and for c-Cbl have distinct phenotypes related to differential expression during T lymphocyte development (31–33, 59). With the range of physical and functional interactions of UIM, Cbl, and WW domain HECT E3 proteins at least partially revealed, a major challenge will be to ascertain the relative significance of individual members of each family in physiological and pathological states.

-- A. Magnifico, C. Yang, J. Mariano, S. Lipkowitz, and A. M. Weissman, unpublished observations.
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