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Interactions of *Drosophila* Cbl with Epidermal Growth Factor Receptors and Role of Cbl in R7 Photoreceptor Cell Development

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The human proto-oncogene product c-Cbl and a similar protein in *Caenorhabditis elegans* (Sli-1) contain a proline-rich COOH-terminal region that binds Src homology 3 (SH3) domains of proteins such as the adapter Grb2. Cbl-Grb2 complexes can be recruited to tyrosine-phosphorylated epidermal growth factor (EGF) receptors through the SH2 domain of Grb2. Here we identify by molecular cloning a *Drosophila* cDNA encoding a protein (*Drosophila* Cbl [D-Cbl]) that shows high sequence similarity to the N-terminal region of human c-Cbl but lacks proline-rich sequences and fails to bind Grb2. Nonetheless, in COS-1 cells, expression of hemagglutinin epitope-tagged D-Cbl results in its coimmunoprecipitation with EGF receptors in response to EGF. EGF also caused tyrosine phosphorylation of D-Cbl in such cells, but no association of phosphatidylinositol 3-kinase was detected in assays using anti-p85 antibody. A point mutation in D-Cbl (G305E) that suppresses the negative regulation of LET-23 by the Cbl homolog Sli-1 in *C. elegans* prevented tyrosine phosphorylation of D-Cbl as well as binding to the liganded EGF receptor in COS-1 cells. Colocalization of EGF receptors with both endogenous c-Cbl or expressed D-Cbl in endosomes of EGF-treated COS-1 cells is also demonstrated by immunofluorescence microscopy. In lysates of adult transgenic *Drosophila melanogaster*, GST-DCbl binds to the tyrosine-phosphorylated 150-kDa torso-DER chimeric receptor. Expression of D-Cbl directed by the *sevenless* enhancer in intact *Drosophila* compromises severely the development of the R7 photoreceptor neuron. These data suggest that despite the lack of Grb2 binding sites, D-Cbl functions as a negative regulator of receptor tyrosine kinase signaling in the *Drosophila* eye by a mechanism that involves its association with EGF receptors or other tyrosine kinases.

The human proto-oncogene product c-Cbl is a 908-amino-acid (aa) protein containing a ring finger domain and an extended proline-rich COOH terminus with multiple PXXP motifs that bind proteins having Src homology 3 (SH3) domains, including Grb2 and Nck (4, 9, 12, 21, 23, 25, 28). Activation of hematopoietic cells leads to rapid tyrosine phosphorylation of c-Cbl and the recruitment of p85/phosphatidylinositol 3-kinase (PI 3-kinase) and Crk to tyrosine phosphorylation sites on Cbl (6, 8, 9, 12, 14, 21, 23, 25, 26, 28). In other cell types, Cbl becomes tyrosine phosphorylated in response to a variety of extracellular signals, including epidermal growth factor (EGF), nerve growth factor, granulocyte-macrophage colony-stimulating factor, and transforming growth factor α (9, 11, 13, 19, 22, 23). Rapid association of c-Cbl with autophosphorylated EGF and colony-stimulating factor 1 (CSF-1) receptors also occurs (5, 13, 22, 32). A mechanism by which Cbl is recruited to the EGF receptor in response to EGF appears to involve binding of the SH2 domain of the adapter Grb2 to tyrosine-phosphorylated sites on the receptor (22, 32). The oncogenic form, v-Cbl, is a truncated 40-kDa protein lacking the ring finger and proline-rich COOH-terminal domain (3, 17) that mediates potent signals leading to cell transformation. A recent report has shown that v-Cbl is poorly tyrosine phosphorylated upon EGF stimulation but nonetheless is recruited to the liganded EGF receptor (5). These and other data argue that the N-terminal regions of c-Cbl (13) and v-Cbl (5) may also interact directly with the EGF receptor.

The physiological functions of c-Cbl are poorly understood. The binding of Grb2 and other proteins to c-Cbl suggests that it may serve as a docking protein for adapter molecules to enhance the formation of protein complexes on activated receptors at the cell membrane. Genetic data obtained with Sli-1, a *Caenorhabditis elegans* protein similar to c-Cbl, indicates a potential suppressor role in signaling by LET-23, the EGF receptor homolog (35). Thus, expression of Sli-1 in the presence of a defective LET-23 receptor prevented vulva development, which is dependent on the $p21^{ras}$ signaling pathway (24). These data suggest the hypothesis that c-Cbl negatively modulates EGF receptor signaling and that v-Cbl may act in a dominant inhibitory mode to block the negative influence of c-Cbl. To clarify the role(s) of Cbl proteins in cellular signaling processes, we searched for similar proteins expressed in *Drosophila melanogaster*. Here we report the molecular cloning of a gene encoding *Drosophila* Cbl (D-Cbl); we show that this protein lacks the entire C-terminal proline-rich domain of mammalian Cbl and does not bind p85 or Grb2. Despite the lack of these sites, D-Cbl is rapidly recruited to EGF receptors following EGF stimulation when heterologously expressed in

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mammalian cells and exerts a negative influence on the signaling pathway required for R7 photoreceptor neuron development in intact *Drosophila*.

MATERIALS AND METHODS

Cloning of D-Cbl. Degenerate primers were made based on two regions, TYDEVK and YELYCEM, that share 100% amino acid sequence identity among the human, mouse, and *C. elegans* homologs of Cbl. These primers were used to amplify a 309-bp DNA fragment from *Drosophila* genomic DNA. This fragment was subcloned, and sequence analysis confirmed that it contained an open reading frame highly homologous to those encoding Cbl proteins. A *Drosophila* eye-specific cDNA library (gift of G. M. Rubin) was screened by using the 309-bp fragment as a probe, and nine independent clones were isolated. The longest cDNA, a 2.66-kb insert, was entirely sequenced with a model 373A DNA sequencer (Applied Biosystems Inc.), using dye-coupled terminators.

Generation of transformants. The D-Cbl cDNA was subcloned as an *Eco*RI fragment into a modified Sev-S11 vector (gift of K. Basler and E. Hafen) containing two copies of the sevenless enhancer (sE) and an *hsp70* promoter. The resulting transformation construct was named sE[D-Cbl]. To generate transformant flies, *Drosophila* w^{1118} females were crossed to males containing P(D2-3) as a source of transposase. The embryos from this cross were injected with sE[D-Cbl], giving rise to flies carrying a D-Cbl transgene.

***Drosophila* binding.** Flies carrying a chimeric *Drosophila* torso-EGF receptor on the X chromosome controlled by an *hsp70* promoter (27) were provided by Ernst Hafen (Zurich, Switzerland) and by Jean Olivier (Toronto, Ontario, Canada). Flies were heat shocked at 37°C for 45 min, allowed to recover at 22°C for 2.5 h, and lysed in 1% Triton X-100–10% glycerol–150 mM NaCl–20 mM Tris (pH 7.5)–1.5 mM Mg^{2+}–1 mM EGTA containing 1 mM Na_2VO_4, 2.5 μg of aprotinin per ml, and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation, clarified supernatants were incubated with 2 μg of glutathione *S*-transferase (GST) or GST-DCbl for 1 h at 4°C, washed twice in 0.2% Triton X-100 buffer and once in 0.2% Triton X-100 buffer containing 0.5 M NaCl, and boiled in sodium dodecyl sulfate (SDS) sample buffer, and proteins were resolved by SDS-gel electrophoresis.

Antibodies and reagents. The *Drosophila* cDNA of 2.66 kb was amplified by PCR with *Taq* polymerase to yield an N-terminal *Eco*RI-methionine-*Nco*I 0.66-kb fragment, using oligonucleotides 5′-GAATTCATGGCGACGAGAGG CAGT-3′ (sense) and 5′-GTGGTCTTTAGGGCCAT-3′ (antisense), and a C-terminal *Nco*I-*Bam*HI 0.76-kb fragment, using oligonucleotides 5′-TAATCTCC GGCCTGGAG-3′ (sense) and 5′-GCGGATCCTGTGAACAAAACTATACC -3′ (antisense). Both PCR products were ligated into *Eco*RI-*Bam*HI-restricted pCMV5. Next, an epitope tag containing a methionine start codon and three tandem copies of the hemagglutinin (HA1) epitope (34) was ligated in frame into the *Eco*RI site at the N terminus of D-Cbl, and the 1.5-kb cDNA was verified by sequencing. The G305E point mutation was generated by ligating a *Cla*I-*Bst*UI 211-bp fragment and a *Bst*UI-*Pst*I double-stranded oligonucleotide containing the desired mutation into pCMV5-HA D-Cbl that was restricted with *Cla*I and *Pst*I to remove the *Cla*I-*Pst*I fragment. The resulting plasmid DNA was sequenced to confirm the mutation. A full-length D-Cbl–GST fusion protein was generated by amplification of D-Cbl cDNA with Vent polymerase (New England Biolabs) and ligation of the product in frame into the *Bam*HI-*Eco*RI site of pGEX2T. Fusion proteins were affinity purified on glutathione-agarose beads (Sigma) according to standard procedures. An N-terminal human Cbl-GST fusion protein, consisting of aa 26 to 283, was prepared by PCR amplification of the human Cbl cDNA with primers 5′-GATTGGGATCCTGAAGGACGC-3′ (sense) and 5′-GAATTTCTGGATCCGAGCTTTC-3′ (antisense). The GST–N-Cbl cDNA was cloned into pGEX2T and sequenced to ensure fidelity of amplification, and the expressed fusion protein was injected into rabbits. The polyclonal antibody (R4575) quantitatively precipitates Cbl from lysates of COS cells. Polyclonal Cbl antibody for Western blots was purchased from Santa Cruz, Inc., and EGF receptor antibody was a gift from Roger Davis, Howard Hughes Medical Institute, University of Massachusetts Medical Center. An anti-HA antibody (R4289) was raised in rabbits against a peptide corresponding to the HA epitope and purified by binding to protein A-Sepharose. An anti-HA monoclonal antibody (MAb) (12CA5) used to blot was purchased from Berkeley Antibody Co. Polyclonal anti-p85 antibody was purchased from Upstate Biotechnology, Inc., and anti-Grb2 MAb was purchased from Transduction Laboratories. A polyclonal Grb2 antibody was produced as described previously (21). A phosphopeptide corresponding to the likely p85 binding site on Cbl (SCT pYEAMYNIQSQA) was synthesized in the Peptide Synthesis Facility at the University of Massachusetts Medical Center. The peptide was coupled to Affi-Gel 10 as instructed by the manufacturer (Bio-Rad).

Cell culture and activation. COS-1 cells were grown in 10-cm-diameter plates in Dulbecco's modified Eagle's medium containing 8% fetal calf serum. Cells were transfected with 1 μg of cDNA in Lipofectamine (GIBCO) according to the manufacturer's protocol. After 2 days, subconfluent cells were serum starved for 4 h and activated at 37°C with 300 ng of EGF (Preprotec) per ml for the indicated times. Reactions were stopped with cold phosphate-buffered saline (PBS) containing 0.5 mM Na_2VO_4, 5 mM EDTA, and 10 mM NaF and lysed in a mixture containing 1% Nonidet P-40 (NP-40), 10 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1 mM Na_2VO_4, 0.1 mM phenylmethylsulfonyl fluoride, and 2.5 μg of aprotinin per ml.

Immunofluorescence microscopy. COS-1 cells were seeded on coverslips and transfected with Lipofectamine and after 2 days were serum starved. EGF (300 ng/ml) was added for 20 min; the cells were washed in cold PBS and fixed in cold 4% formaldehyde. Cells were permeabilized and blocked in PBS containing 1% fetal bovine serum and 0.5% Triton-X 100 for 20 min. Primary antibodies were sheep anti-EGF receptor, rabbit anti-Cbl, and rabbit anti-HA; secondary antibodies were donkey anti-rabbit–fluorescein isothiocyanate (FITC; Pierce) or donkey anti-sheep–rhodamine (Cortex Biochemicals). Cells were mounted in 90% glycerol–PBS–2.5% 1,4-diazabicyclo-(2,2,2)-octane, and images were taken with a digital imaging microscope (Nikon Diaphot 200) fitted with a Nikon Apo60/1.4 immersion lens.

Immunoprecipitation and blotting. Antibodies were mixed with clarified lysates containing 0.5 mg of protein and incubated at 4°C overnight. Protein A-Sepharose was added for 15 min with constant shaking; beads were subsequently washed several times in 0.2% NP-40 buffer and once in 0.2% NP-40 buffer containing 0.5 M NaCl. Proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted. Detection was by chemiluminescence (Boehringer Mannheim).

Nucleotide sequence accession number. The nucleotide sequence of *Drosophila* Cbl has been submitted to GenBank and given accession number U87925.

RESULTS

Isolation of a *Drosophila* cDNA related to the proto-oncogene c-*cbl*. A D-*cbl* DNA fragment was PCR amplified from *Drosophila* genomic DNA by using a set of degenerate primers. Screening of a *Drosophila* eye disc cDNA library with this fragment led to the isolation of a 2.66-kb cDNA containing 313 nucleotides of 5′ noncoding region, a 1,344-nucleotide open reading frame preceding an in-frame TAG stop codon, and 1,317 nucleotides of 3′ noncoding region. A comparison of the derived amino acid sequences of human-Cbl, D-Cbl, and *C. elegans* Cbl (Sli-1) (Fig. 1A) shows no homology over the first 40 aa or beyond residue 426 of D-Cbl. However, within the conserved region of approximately 380 aa, the three species show 63% similarity between aa 46 and 205 and 93% similarity between aa 205 and 330. Within the N-terminal conserved region, several tyrosine residues found in human Cbl are not present in D-Cbl, including the human Y^{141}EEN sequence (F^{129}EDN in D-Cbl), which is a potential Src kinase SH2 binding motif. A high degree of similarity is also observed between aa 354 and 425. This region includes a ring finger domain and a sequence of 17 aa (called 70Z/3) which, when deleted, renders c-Cbl transforming (1, 3, 4). Interestingly, D-Cbl is only 93 residues longer than v-Cbl (Fig. 1B), but these amino acids include the important 70Z/3 sequence and ring finger regions. Finally, unlike the protein found in *C. elegans* or mammals, D-Cbl contains no proline-rich motifs.

D-Cbl negatively regulates R7 development. In the developing *Drosophila* eye, the differentiation of photoreceptor neurons (R cells) depends on signaling through the Sevenless and EGF receptor tyrosine kinases (reviewed in reference 36). The developing eye constitutes a genetic system where the in vivo function of *Drosophila* Cbl could be studied. Mutations in the D-*cbl* locus have not yet been identified, and the region encoding this locus is not covered by deficiencies or transposable elements. We therefore generated transformant flies carrying the D-Cbl cDNA under the transcriptional control of sE, which will cause the expression of the D-Cbl cDNA in all cells that express Sevenless, including the R7 photoreceptor precursor (2). To assess the role of D-Cbl in R7 development, we tested these transgenic flies in a sensitized genetic assay (reviewed in reference 7). In this assay, signaling through Sevenless is compromised by using a disabled Sevenless kinase, sev^{E4}. This defect is partially rescued by combining sev^{E4} with one copy of Sos^{JC2}, an allele of the *son of sevenless* gene encoding an overactive protein. In the resulting sev^{E4}/Y; Sos^{JC2}/+ fly, the re-

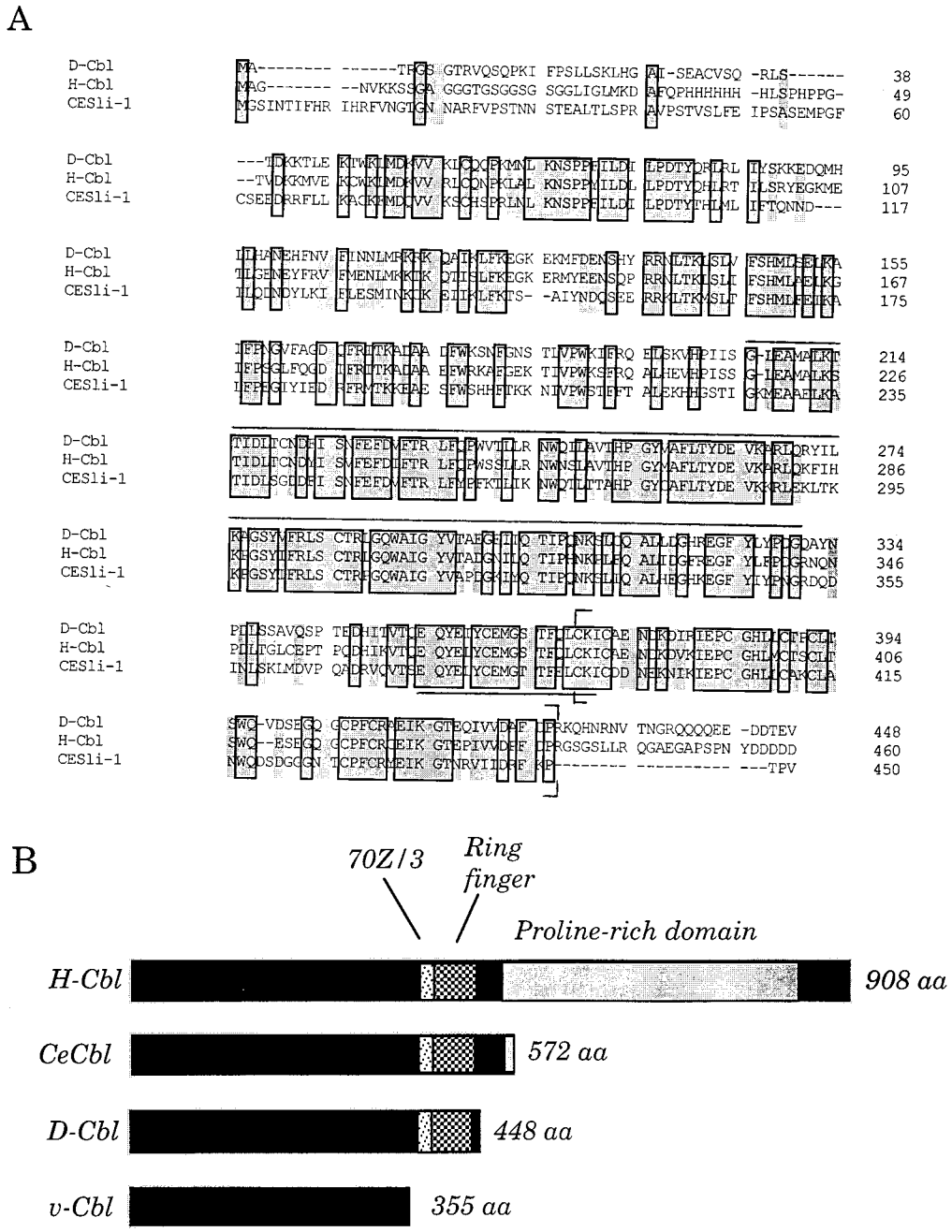

FIG. 1. (A) Comparison of the amino acid sequences of D-Cbl with those of human Cbl (H-Cbl) and *C. elegans* Cbl (CESli-1). The most conserved region of the three proteins, from residues 205 to 330 of dCbl, is overlined. The 70Z/3 17-aa sequence is underlined, and the ring finger domain is boxed. (B) Schematic representation of human c-Cbl, D-Cbl, *C. elegans* Cbl (CeCbl), and v-Cbl.

ceptor tyrosine kinase signal is weakened so that an R7 cell develops in only 17% of the facets (ommatidia) in the eye (Fig. 2).

The above-described genetic background is extremely sensitive to the dosage of genes participating in receptor tyrosine kinase signaling, and the fraction of ommatidia developing R7 cells is a readout for the strength of the transduced signal. We have previously shown that removing a single copy of a positive regulator such as Ras or Sos abolishes the development of R7 cells in this genetic background, while removing one copy of a negative regulator, such as GAP1, will increase this number significantly (29). Removing a copy of the EGF receptor in this background also completely abrogates R7 differentiation (Fig. 2). This finding suggests that the R7 developmental readout is due to the combination of Sev and EGF receptor tyrosine kinase signals. This result is consistent with the recent observations of Freeman (10) showing that both of these receptors are essential for R7 development. We introduced a copy of the D-Cbl transgene in the sensitized background to generate a fly of the genotype sev^{E4}/Y; Sos^{JC2}/+; *sE*[*D-Cbl*]. In the three independently generated transgenic lines shown, the development of R7 cells was essentially eliminated when the level of

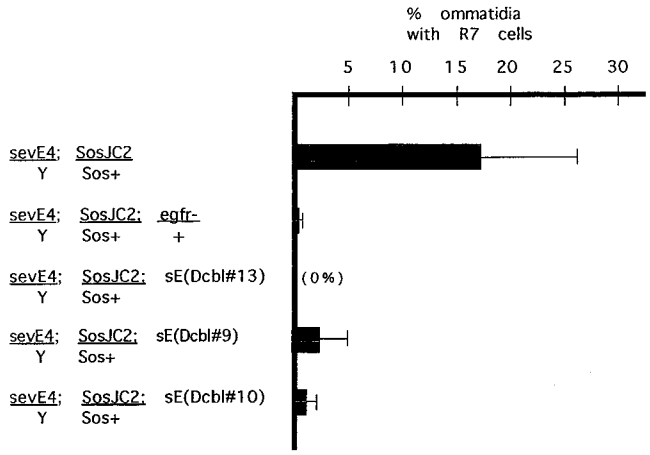

FIG. 2. D-Cbl is a negative regulator of R7 development. The number of developing R7 cells in a sev^{E4}/Y; Sos^{JC2}/+ genetic background is sensitive to a single-copy loss-of-function mutation in the EGF receptor (egfr) and to the sE[D-Cbl] transgene. The eyes were scored by the pseudopupil technique, and for each genotype at least 2,000 ommatidia were counted.

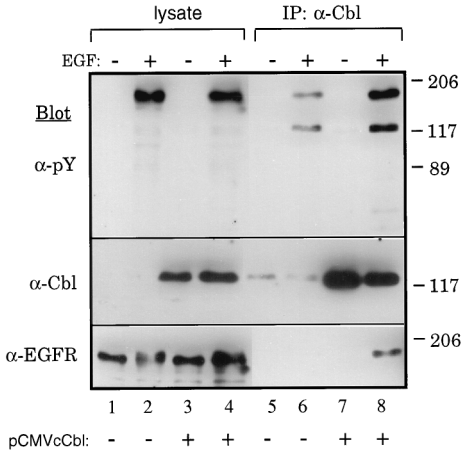

FIG. 4. Human c-Cbl is recruited to the EGF receptor upon EGF stimulation of transiently transfected COS-1 cells. Cells containing endogenous Cbl (lanes 1, 2, 5, and 6) or transfected with c-Cbl (lanes 3, 4, 7, and 8) were activated with EGF (310 ng/ml) for 20 min. Lysates were prepared, and 500 μg of protein was immunoprecipitated with anti-Cbl (α-Cbl) and blotted with antiphosphotyrosine (α-PY; top), anti-Cbl (middle), or anti-EGF receptor (α-EGFR; bottom). Lanes 1 to 4, 10 μg of total cell lysate. Sizes are indicated in kilodaltons.

D-Cbl was raised in the R7 precursors (Fig. 2). No change in phenotype was observed when D-Cbl was added to flies with a wild-type genetic background (data not shown). This result strongly argues that in the *Drosophila* eye, D-Cbl acts as a negative regulator of one or more receptor tyrosine kinase pathways that are essential for photoreceptor differentiation.

D-Cbl does not bind p85 or Grb2. Since D-Cbl does not contain any of the proline-rich domain found in the *C. elegans* Sli-1 homolog or in mammalian Cbl (Fig. 1) and has only one (Y^{370}CEM) of two possible p85 binding sites, we tested whether Grb2 or p85 was bound to D-Cbl. This was accomplished by heterologously expressing D-Cbl or human c-Cbl in COS-1 monkey kidney cells and activating with EGF. COS-1 cells were transiently transfected with HA epitope-tagged D-Cbl cDNA or human c-Cbl cDNA 48 h prior to lysis and immunoprecipitation with anti-c-Cbl or anti-HA antibody. Immunoblots of anti-HA precipitates with anti-p85 showed no detectable p85 associated with D-Cbl (Fig. 3, lanes 5 and 6), consistent with the lack of the Y^{731}EAM motif in D-Cbl. In COS cells transfected with c-Cbl cDNA, an EGF-dependent binding of p85 to Cbl is observed, as expected (Fig. 3, lanes 3 and 4), and immobilized phosphopeptide containing the c-Cbl SH2 recognition motif (Y^{731}EAM) for p85 binds a similar amount of p85 (Fig. 3, lane 7). These data indicate that the p85 concentration in these lysates is not rate limiting for binding to D-Cbl and that the Y^{731}EAM motif is phosphorylated by EGF and binds p85 in these cells. The Grb2 immunoblot (Fig. 3, middle panel) of the anti-HA and anti-Cbl immunoprecipitates shows that Grb2 is bound to Cbl in c-Cbl-transfected cells (Fig. 3, lanes 3 and 4) but is not associated with D-Cbl (Fig. 3, lanes 5 and 6). Interestingly, activation of COS-1 cells with EGF causes more Grb2 to be complexed with Cbl (Fig. 3, lane 4), consistent with an increased Grb2/Cbl stoichiometry observed in activated Jurkat T cells (21), as well as EGF-activated 293 kidney (22) and human mammary epithelial cells (12).

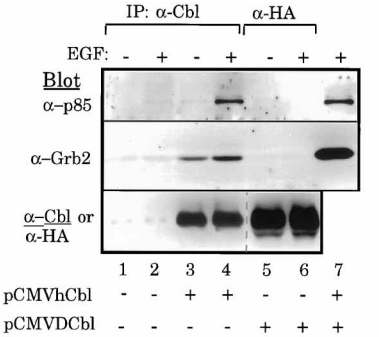

FIG. 3. Grb2 and p85 do not associate with D-Cbl. COS-1 cells were transfected with c-Cbl (lanes 3 and 4) or HA-DCbl cDNA (lanes 5 and 6) and activated with EGF for 5 min. Lysates containing 500 μg of protein were immunoprecipitated (IP) with anti-Cbl (α-Cbl; lanes 1 to 4) or anti-HA (α-HA; lanes 5 and 6). A p85 phosphopeptide coupled to Affi-Gel (lane 7, top panel) or anti-Grb2 coupled to protein A-Sepharose (lane 7, middle panel) was added to lysates from EGF-activated cells. After electrophoresis, nitrocellulose filters were blotted with the indicated antibody.

Recruitment of D-Cbl to the EGF receptor. c-Cbl is recruited to the EGF receptor in mammalian cells following EGF stimulation (5, 13, 22, 32) and appears to negatively regulate EGF receptor and/or Sevenless signaling in *Drosophila* (Fig. 2). In *C. elegans*, the 582-residue Sli-1 homolog of c-Cbl appears to be a negative regulator of the EGF receptor homolog, LET-23 (35). We therefore examined whether HA epitope-tagged D-Cbl expressed in COS-1 cells becomes associated with the EGF receptor. Control experiments were first conducted with c-Cbl in the COS-1 cell system to ensure that EGF stimulation of these cells causes formation of EGF receptor–c-Cbl complexes. Figure 4 shows that expression of c-Cbl (lane 3 and 4 or 7 and 8) is indeed accompanied by an EGF-dependent increase in a tyrosine phosphorylated p170 bound to c-Cbl immunoprecipitates (top [compare lanes 6 and 8]) and that p170 immunoreacts with EGF receptor antibody (bottom, lane 8). Complexes of EGF receptor and endogenous c-Cbl in nontransfected cells are also readily observed in these experiments (top, lanes 5 and 6). Unlike the transient phosphorylation of c-Cbl in response to T-lymphocyte activation (9, 21), tyrosine phosphorylation of the EGF receptor and Cbl in COS-1 cells is more stable, exhibiting a half-life of 2 h (not shown).

Transfection of COS-1 cells with the HA epitope-tagged D-Cbl cDNA (Fig. 5) revealed a p60 polypeptide correspond-

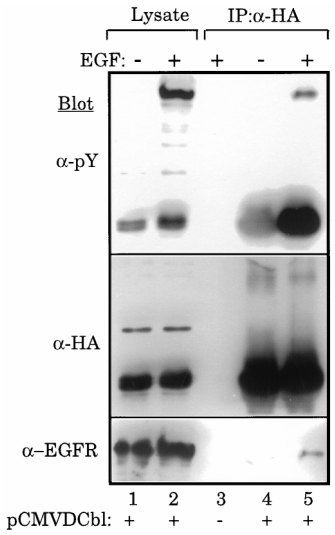

FIG. 5. EGF stimulates recruitment of D-Cbl to the EGF receptor in transiently transfected COS-1 cells. Cells were transfected with HA-DCbl and 48 h later serum starved for 2 h. Cells were activated with EGF (310 ng/ml), and detergent-soluble lysates were immunoprecipitated (IP) with anti-HA (α-HA). Proteins bound to nitrocellulose filters were blotted with antiphosphotyrosine (α-PY; top), anti-HA (middle), or anti-EGF receptor (α-EGFR; bottom). Lanes 1 and 2, 10 μg of total cell lysates; lanes 3 to 5, anti-HA immunoprecipitates. Lane 3, lysates of nontransfected cells immunoprecipitated with anti-HA.

ing to the expected migration of D-Cbl in lysates and in anti-HA immunoprecipitates of transfected cells that was immunoreactive with anti-HA MAb. This polypeptide band was not found in anti-HA immunoprecipitates of nontransfected cells (Fig. 5, lane 3). In nonstimulated cells, D-Cbl was tyrosine phosphorylated (lanes 1 and 4), and activation with EGF led to increased phosphorylation (lanes 2 and 5), as well as the appearance of p170 in anti-HA immunoprecipitates (top, lane 5). Blotting anti-HA immunoprecipitates of COS-1 cells transfected with D-Cbl cDNA with anti-EGF receptor (bottom, lane 5) showed that D-Cbl is recruited to the 170-kDa EGF receptor in an activation-dependent manner. These results were confirmed by identifying D-Cbl in immunoprecipitates of the EGF receptor (Fig. 6). When EGF receptors were immunoprecipitated from D-Cbl-transfected cells and immunoblotted with anti-HA antibody, a small amount of D-Cbl was present. EGF addition to the COS-1 cells increased this association of D-Cbl with EGF receptors several fold (lanes 5 and 6).

A Gly^{315}-to-Glu point mutation in the *C. elegans* homolog of

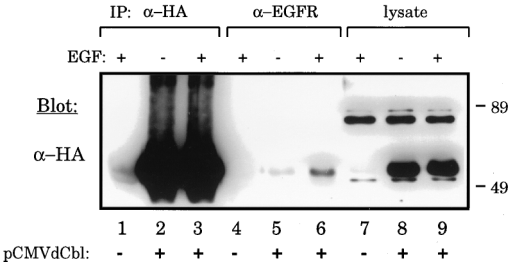

FIG. 6. Immunoprecipitates of COS-1 cell EGF receptors contain D-Cbl. Cells were transfected with HA-DCbl as noted and activated for 20 min with EGF, and 0.5 mg of detergent-soluble lysates was immunoprecipitated with anti-HA (α-HA; lanes 1 to 3) or anti-EGF receptor (α-EGFR; lanes 4 to 6). Lanes 7 to 9, 10 μg of total cell lysates. Sizes are indicated in kilodaltons.

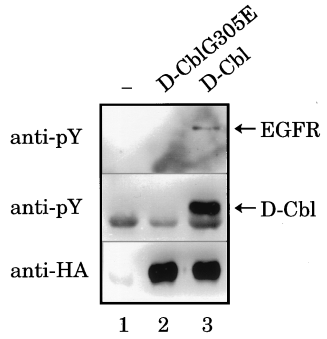

FIG. 7. A G305E point mutation in D-Cbl prevents binding to the liganded EGF receptor (EGFR) in COS-1 cells. Cells were transfected with HA-DCbl (lane 3) or HA-G305E-DCbl (lane 2) and activated with EGF, and lysates were immunoprecipitated with anti-HA. Proteins bound to nitrocellulose filters were blotted to anti-HA (bottom) or antiphosphotyrosine (anti-PY).

Cbl, Sli-1, suppresses the ability of this protein to act as a negative regulator of LET-23 signaling (35). We therefore tested whether the association of D-Cbl and the EGF receptor was also prevented by the analogous mutation in D-Cbl. In Fig. 7, COS-1 cells were transfected with D-Cbl or the corresponding G305E D-Cbl point mutation, and after stimulation with EGF, detergent-soluble lysates were immunoprecipitated with anti-HA. The anti-HA blot shows that although the wild-type and mutant proteins were expressed to the same extent (Fig. 7, bottom, lanes 2 and 3), only D-Cbl was bound to the EGF receptor (top, lane 3). Importantly, the G305E mutation of D-Cbl totally prevented tyrosine phosphorylation of the expressed protein by EGF (middle, lanes 2 and 3). We conclude that the loss-of-function mutation in *C. elegans* acts by preventing the association of Cbl with the EGF receptor.

The association of transiently expressed D-Cbl with liganded EGF receptors in COS-1 cells was confirmed in *Drosophila* by in vitro binding of a D-Cbl fusion protein to extracts of flies expressing a chimeric torso-DER receptor. The chimeric receptor consists of the extracellular domain of a mutant torso receptor fused to the cytoplasmic domain of DER under the control of the *hsp70* heat shock promoter (27). In Fig. 8, heat shock of adult transgenic *Drosophila* for 45 min at 37°C, followed by a recovery at 22°C for 3 h, causes a pronounced tyrosine phosphorylation of many proteins (lanes 1 and 2).

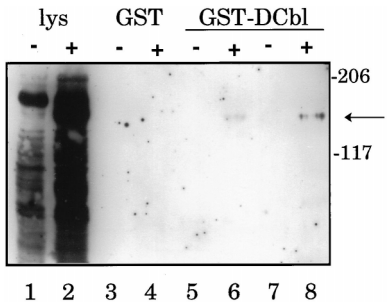

FIG. 8. D-Cbl binds in vitro to the *Drosophila* EGF receptor. Adult *Drosophila* flies transfected with a torso-DER chimeric receptor were heat shocked for 45 min. After recovery, lysates from control (−) and heat-shocked (+) flies were prepared in a 1% Triton X-100 buffer, and 0.5 (lanes 5 and 6) or 1.0 (lanes 7 and 8) mg of protein was bound to 2 μg of GST-DCbl. Washed complexes bound to glutathione-agarose were electrophoresed, transferred to nitrocellulose, and blotted with antiphosphotyrosine. In lanes 3 and 4, 2 μg of GST protein was bound to 1 mg of cell lysate from heat-shocked flies. Lanes 1 and 2, 20 μg of total cell lysates (lys). Sizes are indicated in kilodaltons.

Addition of GST–D-Cbl fusion protein to 0.5 mg (lanes 5 and 6) or 1.0 mg (lanes 7 and 8) of cell lysate resulted in the binding of a 150-kDa protein in extracts of flies subjected to heat shock but not to control flies. No tyrosine-phosphorylated bands appeared in lysates reacted with a GST fusion protein (lanes 3 and 4). A GST-Grb2 fusion protein also binds strongly to a 150-kDa protein in extracts of activated flies (data not shown), confirming that p150 is DER.

Colocalization of D-Cbl and human c-Cbl with the EGF receptor. The biochemical evidence indicating a ligand-dependent association of human c-Cbl and D-Cbl with the EGF receptor (Fig. 4 to 6) was further evaluated by examining the subcellular localization of these proteins after EGF stimulation. For this purpose, COS-1 cells were grown on coverslips and incubated with or without EGF for 20 min before washing and fixing in cold formaldehyde. COS-1 cells transfected with D-Cbl cDNA were double stained with anti-HA antibody (visualized with FITC-labeled secondary antibody) and anti-EGF receptor antibody (visualized with rhodamine-labeled secondary antibody). Endogenous c-Cbl in nontransfected cells was detected with anti-c-Cbl antibody and visualized with FITC-labeled secondary antibody. Prior to the addition of EGF, endogenous EGF receptor and c-Cbl appear diffuse (Fig. 9C and D), whereas 20 min after EGF addition, the EGF receptor becomes internalized, as evidenced by the punctate rhodamine staining (Fig. 9F). Visualization of the same cell with anti-Cbl (Fig. 9E) shows that a portion of endogenous c-Cbl becomes colocalized with the internalized EGF receptor. COS cells that overexpress Cbl show the same pattern, although the greater intensity of Cbl staining throughout the cell obscures the punctate signal to some degree (data not shown).

Immunofluorescence microscopy staining of D-Cbl-transfected COS cells reveals a similar pattern of colocalization of D-Cbl with the EGF receptor in EGF-activated cells. Thus, in serum-starved cells, D-Cbl appears to be diffuse (Fig. 9A), whereas 20 min after addition of EGF, a fraction of cellular D-Cbl becomes concentrated in EGF receptor-enriched vesicles (Fig. 9G and H). We conclude that EGF causes D-Cbl and c-Cbl to be translocated into internalized vesicles containing the EGF receptor, consistent with the physical association of the Cbl proteins with EGF receptors (Fig. 4 and 5).

DISCUSSION

Signaling events initiated by receptor tyrosine kinases are crucial for establishing the identity of photoreceptor neurons in the developing *Drosophila* eye. We have molecularly characterized D-Cbl, the *Drosophila* homolog of the c-Cbl oncogene product. Genetic experiments have demonstrated that in flies expressing elevated levels of D-Cbl in the R7 precursor, the development of this cell as a photoreceptor neuron is compromised (Fig. 2). This finding strongly suggests that D-Cbl is normally involved in the negative regulation of receptor tyrosine kinase-mediated signals. These results, as well as those of Freeman (10), suggest that in addition to Sevenless signaling, EGF receptor activity is also required for R7 cell fate determination. Therefore, the negative regulatory function of D-Cbl on R7 cell differentiation reflects its participation either in EGF receptor signaling or in Sevenless signaling, or possibly in both. This genetic interaction between D-Cbl and the receptor tyrosine kinase pathway is consistent with our biochemical analysis showing that D-Cbl binds to the EGF receptor (Fig. 4 to 6).

A major finding in this work is that D-Cbl is devoid of a COOH-terminal domain containing proline-rich Grb2 binding motifs. Much of the previous work on the proto-oncogene c-Cbl protein has focused on identification of signaling proteins that associate with its proline-rich motifs through SH3 binding or its tyrosine phosphate sites through SH2 binding. These associated proteins include *Src* family tyrosine kinases, Grb2, Nck, Crk-G3G complexes, and PI3 kinases (8, 9, 14, 21, 23, 25, 26, 28, 30). Interestingly, the motifs thought to bind all of these associated proteins are believed to reside in the proline-rich COOH-terminal region of c-Cbl containing residues 480 to 850. The recently identified *C. elegans* Cbl homolog, Sli-1, lacks much of this COOH-terminal domain but does contain several proline-rich motifs between aa 470 and 570 that can serve as binding sites for the adapter Grb2. We confirmed that D-Cbl does not bind Grb2 or PI3 kinases upon its transient expression in COS-1 cells (Fig. 3). Thus, our data indicate that D-Cbl can function in *Drosophila* without the Grb2 homolog Drk or the many other signaling proteins that bind this region in c-Cbl.

The binding of c-Cbl to Grb2 through the adapter's N-terminal SH3 domain appears to mediate the recruitment of c-Cbl to activated EGF receptors through binding of the Grb2 SH2 domain to receptor tyrosine phosphorylation sites. However, D-Cbl, which has high sequence similarity to the N-terminal domain of c-Cbl, does retain the ability to be recruited to EGF receptors upon EGF activation of COS-1 cells (Fig. 4 to 6). We have also demonstrated that the tyrosine-phosphorylated *Drosophila* EGF receptor DER binds to a GST–D-Cbl fusion protein in detergent-soluble lysates of adult flies. Thus, the N-terminal region of Cbl proteins appears to provide a second means of EGF receptor association, as suggested by studies with v-Cbl (5) and the N-terminal region of c-Cbl (13). That this is a direct binding is indicated by far-Western blotting of EGF receptors with a GST fusion protein containing the N-terminal region of c-Cbl (13). Taken together, the data now available are consistent with a key role of this EGF receptor-binding N-terminal domain in the functioning of c-Cbl.

A physiologically relevant functional domain within the N-terminal region of c-Cbl proteins is supported by the approximate 68% amino acid sequence identity of human Cbl to D-Cbl between residues 40 and 426 of D-Cbl. Although the exact residues in Cbl that interact with the liganded EGF receptor have not been identified, we have found that a GST fusion protein encompassing the N-terminal 160 aa of c-Cbl does not associate (not shown). Based on the G305E mutation in D-Cbl that ablates receptor association (Fig. 7), it seems plausible that the most highly conserved region of Cbl (aa 205 to 330) may be sufficient for EGF receptor binding. It is possible that this core region of Cbl binds directly to other tyrosine kinases as well. For example, the N terminus of c-Cbl (aa 1 to 357) binds in vitro to ZAP-70 in activated T cells, and the loss-of-function point mutant G306E largely prevents this interaction (20). In T cells activated through the CD3 receptor, c-Cbl binds directly to the Src kinase Fyn in an activation-dependent manner (33), although the region of Cbl responsible for this interaction was not identified. A tyrosine kinase receptor with similarity to the EGF receptor is the hematopoietic cell CSF-1 receptor, and in the macrophage cell line P388D1, Cbl is recruited to the activated CSF-1 receptor (32). However, it is not established whether this binding is mediated through Grb2 or through the Cbl N-terminal domain, or both. It will be important in future studies to determine what structural motif or motifs in these tyrosine kinases binds D-Cbl and whether such motifs appear in other proteins.

It is striking that the association of EGF receptors with D-Cbl and c-Cbl observed by biochemical techniques can be

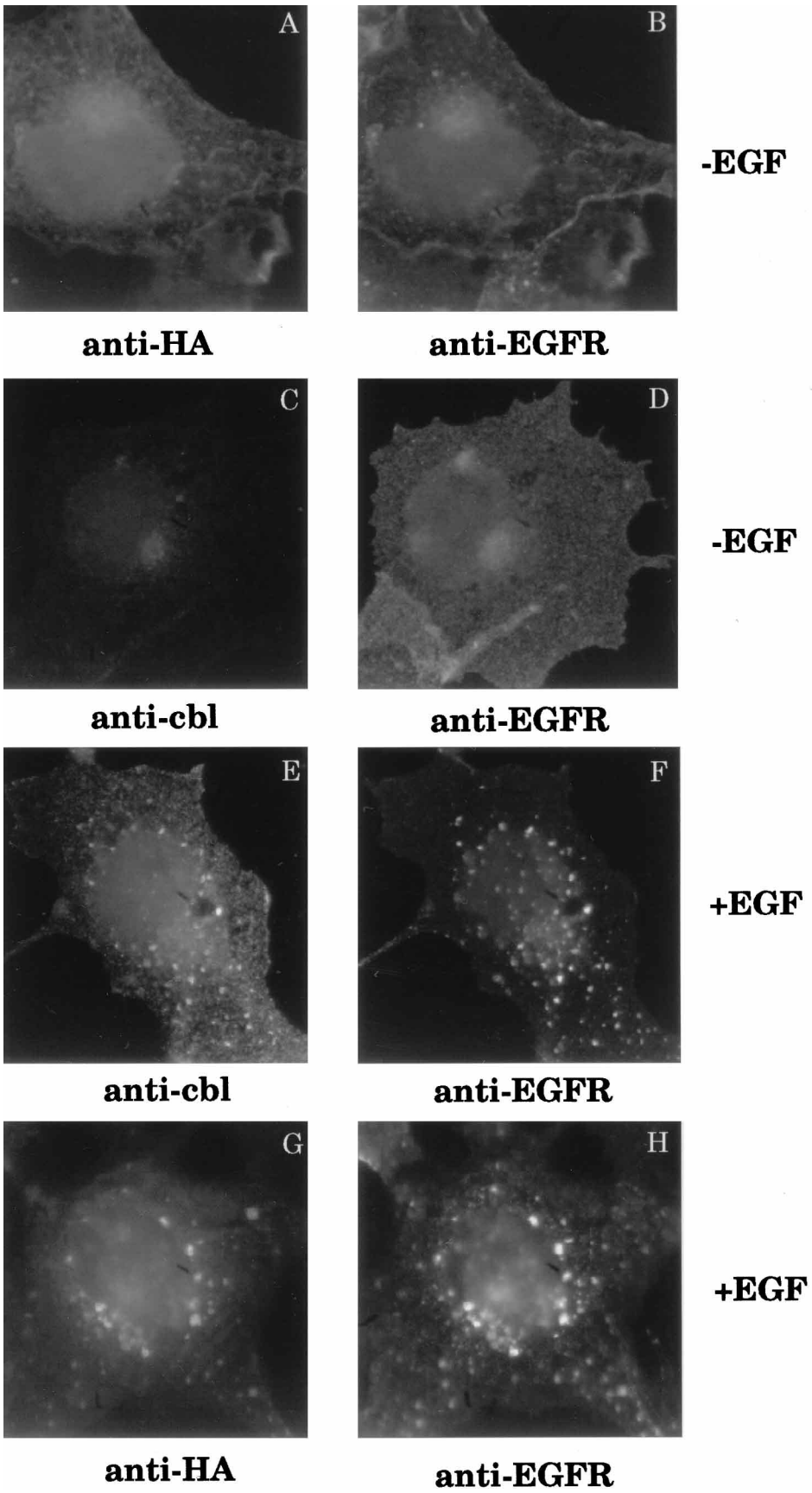

FIG. 9. Colocalization of D-Cbl and c-Cbl with internalized EGF receptors following activation with EGF. Cells were serum starved for 1 h and stimulated for 20 min. with EGF. After fixing and permeabilizing, cells were stained with sheep anti-EGF receptor (anti-EGFR) and donkey anti-sheep–rhodamine. Human Cbl was visualized with rabbit anti-Cbl plus donkey anti-rabbit–FITC; D-Cbl was visualized with rabbit anti-HA plus donkey anti-rabbit–FITC. (C to F) Nontransfected COS-1 cells; (A, B, G, and H) HA-DCbl-transfected cells.

visualized by immunofluorescence microscopy of intact COS-1 cells (Fig. 9). Colocalization of Cbl proteins and EGF receptors can be observed as early as a few minutes after addition of EGF to these cells (not shown), and by 20 min of incubation, an intense punctate pattern of Cbl and the EGF receptor proteins is evident. It is noteworthy that these images can be visualized with endogenous EGF receptors in the COS-1 cells. Our data are consistent with the hypothesis that Cbl-EGF receptor complexes are internalized into endosomes through the coated pit pathway, as previously described for EGF receptors (31). Previously, Tanaka et al. (32) demonstrated by immunofluorescence that Cbl is translocated to a perinuclear region after Fcγ receptor cross-linking or EGF stimulation. The continued association of D-Cbl and c-Cbl proteins with EGF receptors traffiking within intracellular membranes correlates with the known retention of phosphorylated tyrosine residues and active tyrosine kinase activity in the intracellular EGF receptors (15, 16, 31). It seems likely from these observations that modulation of EGF function by Cbl proteins involves the transient association with both cell surface and intracellular EGF receptors. In this context, it is intriguing that mutations in the *sli-1* gene, encoding the *C. elegans* homolog of Cbl, have been shown to interact genetically with *unc-101* in the regulation of the EGF receptor pathway leading to vulval differentiation (18). *unc-101* encodes a clathrin-associated protein homologous to mouse AP47, which constitutes one of the main components of coated pits and vesicles. Although *sli-1* mutations alone are silent, in combination with *unc-101* mutations, they cause excessive vulval differentiation. These gene products, thus, appear to cooperate synergistically in the negative control of EGF receptor signaling. Part of the mechanism whereby Cbl proteins exert an attenuation on receptor tyrosine kinase signaling might involve the downregulation of receptor activity by directing the intracellular degradation of the receptor-Cbl complexes.

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