Evidence for Direct Interaction between Sprouty and Cbl*

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Sprouty (SPRY) was first identified in a genetic screen in Drosophila as an antagonist of fibroblast and epidermal growth factor receptors and Sevenless signaling, seemingly by inhibiting the receptor tyrosine kinase (RTK)/Ras/MAPK pathway. To date, four mammalian Sprouty genes have been identified; the primary sequences of the gene products share a well conserved cysteine-rich C-terminal domain with their Drosophila counterpart. The N-terminal regions do not, however, exhibit a large degree of homology. This study was aimed at identifying proteins with which human SPRY2 (hSPRY2) interacts in an attempt to understand the mechanism by which Sprouty proteins exert their down-regulatory effects. Here, we demonstrate that hSPRY2 associates directly with c-Cbl, a known down-regulator of RTK signaling. A short sequence in the N terminus of hSPRY2 was found to bind directly to the Ring finger domain of c-Cbl. Parallel binding was apparent between the Drosophila homologs of Sprouty and Cbl, with cross-species associations occurring at least in vitro. Coexpression of hSPRY2 abrogated an increase in the rate of epidermal growth factor receptor internalization induced by c-Cbl, whereas a mutant hSPRY2 protein unable to bind c-Cbl showed no such effect. Our results suggest that one function of hSPRY2 in signaling processes downstream of RTKs may be to modulate c-Cbl physiological function such as that seen with receptor-mediated endocytosis.

Receptor tyrosine kinases (RTKs)† have been implicated in numerous cellular processes such as cell fate specification and differentiation (1), oncogenic transformation (2), and axonal guidance (3). Tyrosine residues in the cytoplasmic domains, which become rapidly phosphorylated following ligand engagement, orchestrate the intrinsic properties of RTKs. The activation of RTK (or in some cases, an associated phosphotyrosine-bearing docker protein) thus serves as a docking site that attracts various signaling molecules to the membrane vicinity. The assembly of such signaling complexes allows the RTKs to initiate the transmission of signals from the membrane to the nucleus via the MAPK cascade (4). This pathway uses a set of highly conserved signal transduction molecules to link the activated receptors to the MAPK cascade activator, the GTP-binding protein Ras (5). The increasing number and complexity of proteins discovered to be involved in modulating the MAPK cascade indicate that the transmission of signals originating from the RTKs is under exquisite homeostatic control (6).

In the last decade, a number of major RTK/MAPK regulators have been isolated from genetic screens in developmental models, allowing for the delineation of many key mammalian signaling pathways. Fibroblast growth factor receptors and epidermal growth factor receptors (EGFRs) are subsets of RTKs that are coupled to Ras via one or more adaptor proteins that contain specific protein-protein interaction domains (7, 8). In the EGFR signaling system, one such protein that serves as a direct link between the receptor and Ras is Grb2 (9), which binds constitutively to and recruits the Ras-activating guanine nucleotide exchange factor Sos (10). Ras is subsequently activated by Sos via GDP/GTP exchange. Ras has an inherent, weak GTPase activity. Auxiliary proteins with GTPase-activating properties interact with and enhance the GTPase activity of Ras and hence are down-regulators of MAPK signaling pathways (11). Fibroblast growth factor receptors activate the MAPK cascade by essentially a similar mechanism as EGFR, except they make use of a surrogate receptor cytosolic domain in the form of the constitutively associated FRS2 docker protein (12–14), where tyrosine residues on FRS2 serve as substrates of fibroblast growth factor receptor and attract the SH2 domains of Grb2 and Shp2.

A common component of many signaling modules is the multi-adaptor protein Cbl, first identified as a retroviral transforming gene product that induces pre-B cell lymphoma and myeloid leukemia (15). The product of the mammalian c-cbl gene (p120cbl) is a widely expressed cytoplasmic protein with several distinctive domains, including an SH2 domain, a Ring finger motif, and a large proline-rich stretch at its C terminus (16). Genetic and biochemical studies have implicated c-Cbl in the attenuation of RTK-mediated signaling cascades; partial loss-of-function mutations of Caenorhabditis elegans LET-23 (an EGFR equivalent) result in developmental defects that are reversed by mutation of the c-Cbl ortholog SLI-1, which acts early at the level of LET-23, and the Grb2 homolog Sem5 (15, 17). The engagement of a variety of transmembrane receptors, including growth factor, antigen, and integrin receptors, results in tyrosine phosphorylation of c-Cbl and its association with numerous cytoplasmic signaling proteins (18, 19). Recently, c-Cbl has been demonstrated to elevate the rate of ligand-induced endocytosis (termed “down-regulation”) of EGFR by tagging the receptors with ubiquitin and targeting

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1. The abbreviations used are: RTKs, receptor tyrosine kinases; MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; Grb2, growth factor receptor-binding protein-2; Sos, Son of Sevenless; FRS2, fibroblast growth factor receptor substrate-2; SH2, Src homology domain-2; Ring, really interesting new gene; dSPRY, Drosophila Sprouty; mSPRY, murine Sprouty; hSPRY, human Sprouty; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; dCbl, Drosophila Cbl; GST, glutathione S-transferase; RF, Ring finger; PBS, phosphate-buffered saline; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein ligase; ERK, extracellular signal-regulated kinase.

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them for destruction by the lysosomal/proteasomal system (20).

In *Drosophila*, Sprouty (dSPRY) was first identified as a down-regulator of the “Breathless” (the *Drosophila* equivalent of fibroblast growth factor receptor) signaling cascade that governs proper tracheal branching (21). The removal or loss-of-function mutations of dSPRY gave rise to a morphological “sprouting” effect. dSPRY was also isolated in a separate genetic screen for inhibiting DER (the *Drosophila* equivalent of EGFR)-dependent cell recruitment during eye development (22) and has been reported to be expressed in the developing eye imaginal disc and other tissues where EGFR signaling is known to exert its control (23). The C-terminal half of dSPRY was shown to localize the protein to the inner surface of the plasma membrane of *Drosophila* S2 cells, whereas the N-terminal portion interacted, at least in vitro, with DRK (the *Drosophila* homolog of Grb2) and Gap1, a Ras GTPase-activating protein (22). Sequestration of a docking protein like DRK would constitute a plausible mechanism for inhibition of the early phase of RTK signaling. Gap1 has been shown previously to be a negative regulator of signaling for the *Drosophila* Sevenless RTK (24). It is possible that dSPRY might interact with and augment the GTPase activity of Gap1, thus acting as an attenuator of Ras activity. More recent genetic evidence led to the postulation that dSPRY intercepts the Ras/MAPK cascade downstream of Ras, at the level of Raf or MEK (25). Composite studies thus suggest that dSPRY acts as a general antagonist of the RTK signaling pathways (23, 25).

To date, four mammalian Sprouty homologs have been cloned, with no known binding motifs or physiological functions (21, 26). The Sprouty proteins are classified under the same gene family by virtue of their characteristic cysteine-rich residues located in their carboxyl termini. Parallel investigations on the role of murine SPRY2 (mSPRY2) in the development of the embryonic mouse lung suggest a conservation of function between dSPRY and mSPRY2 with respect to their negative modulation of respiratory organogenesis (27). Furthermore, overexpression of Sprouty constitutes a reduction in fibroblast growth factor-induced limb bud outgrowth (28). We have also recently identified a novel translocation domain that is responsible for the general targeting of Sprouty proteins to membrane ruffles upon fibroblast growth factor/EGF stimulation (29). We were interested in further characterizing hSPRY2 and its possible involvement in RTK signal down-regulation by identifying cellular proteins that interact with hSPRY2. We demonstrate in this study that hSPRY2 and dSPRY interact directly with c-Cbl and dCbl and that the hSPRY2-c-Cbl association leads to an inhibition of the role of c-Cbl in enhancing the rate of internalization and possibly the down-regulation of EGFR.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Monoclonal antibodies to phosphorytrosine (PY20) and EGFR (E12020) and anti-GST polyclonal antibody were purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-c-Cbl (C-15) and murine anti-c-Bl-b (G-1) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein G/protein A-agarose was from Calbiochem. Secondary anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were from Sigma. Anti-Ga4 binding domain and anti-Ga4 activation domain antibodies were from CLONTECH (Palo Alto, CA). Human recombinant EGF was from Upstate Biotechnology, Inc. (Lake Placid, NY). Radioisotopes were from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom).

**DNA Constructs—**hSPRY2 cDNA was amplified by polymerase chain reaction (Expand Long Template polymerase chain reaction system, Boehringer Molecular Biochemicals) from a human brain library (CLONTECH) and subcloned into the pGEX4T1 vector for bacterial expression or into the pXJ40HA and pXJ40FLAG mammalian expression vectors (courtesy of Dr. E. Manser, Glaxo Laboratory, Institute of Molecular and Cell Biology). The N-terminal (residues 1–177), C-terminal (residues 178–315), 30C (residues 30–315), and 51C (residues 53–315) DNA fragments of hSPRY2 (see Fig. SB) and deletion mutants Δ111–53, Δ36–53, Δ36–122, Δ123–177, and Δ178–194 of hSPRY2 (see Figs. 3A and 3B) were generated using standard polymerase chain reaction and molecular cloning methods. *Drosophila* Sprouty cDNA was a kind gift of Dr. G. Martin (University of California). The full-length (residues 1–592), N210 (residues 1–210), 202C (residues 202–592), and Δ178–199 fragments of dSPRY (see Fig. 3D) were subcloned into the pXJ40FLAG vector. Human c-Cbl cDNA was kindly provided by Dr. W. Langdon (University of Western Australia). The full-length c-Cbl (residues 1–906), Cbl-N2 (residues 1–436), Cbl-N3 (residues 1–379), Cbl-N5 (residues 1–290), Cbl-N6 (residues 1–252), Cbl-C10 (residues 437–906), Cbl-C2 (residues 380–906), and Cbl-C3 (residues 362–906) fragments were subcloned into the pXJ40HA vector. The amino acid sequences of full-length c-Cbl, Cbl-N3, and Cbl-C2, were subcloned into the pQE60 vector (courtesy of Dr. B. L. Tang, Institute of Molecular and Cell Biology) for bacterial production of histidine-tagged fusion proteins. The *Drosophila* Cbl expression construct was obtained from Dr. H. Meisner (University of Massachusetts Medical Center), and the full-length (residues 1–450) and ΔRF (residues 1–369) fragments of dCbl (see Fig. 4D) were subcloned into the pXJ40HA vector. The EGFR cDNA was a gift from Dr. A. Ulrich (Max-Planck-Institut fur Biochemie).

**Cell Culture**—293T human kidney epithelial and Chinese hamster ovary cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone Laboratories), 2 mM glutamine, 10 mM HEPES (pH 7.4), and 100 units/ml penicillin/streptomycin. For growth factor–induced signaling and as well as inactivation of c-Cbl, as measured by its susceptibility to destruction by the lysosomal/proteasomal system (20).

**Immunoprecipitation, Pull-down Assays, and Western Blotting—**Protein concentrations of cell lysates were normalized using a BCA protein assay kit (Fermentas) before incubation with 2.5 μg of the appropriate antibody (for immunoprecipitation) or 10 μg of GST fusion protein (for pull-down assays) overnight at 4 °C. Subsequently, 30 μl of protein G/protein A-agarose beads were added to capture the immunocomplex for 1 h. Eluted proteins were resolved on SDSPAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h in PBS containing 1% bovine serum albumin and incubated for 1 h with 1 μg/ml primary antibody followed by 0.5 μg/ml secondary antibody linked to horseshadish peroxidase. Immunoreactive protein bands were detected using the ECL chemiluminescence reagent (Amersham Pharmacia Biotech). A monoclonal antibody (OctA-Probe, Santa Cruz Biotechnology) was used to stain sections of E16.5 embryos. Mouse monoclonal antibodies to c-Cbl (1–189) and c-Cbl (C-15) were generated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and seeded in 6-well plates containing sterilized glass coverslips. At 70% confluence, cells were transfected with 1–2 μg of plasmid DNA using LipofectAMINE™ 2000 reagent (Life Technologies, Inc.). At 4 h post-transfection, the transfection medium was aspirated and replaced with complete medium overnight, followed by incubation in serum-free medium for a further 16 h. Nonadherent or dissociation–quiescent cells were either left untreated or were stimulated with 100 ng/ml EGF at 37 °C for 10 min. Cells were subsequently rinsed with cold PBS/BSA (containing 10 mM calcium chloride and 10 mM magnesium chloride) and fixed with 3% paraformaldehyde in PBS/BSA at 4 °C for 30 min. Cells on coverslips were permeabilized with 0.1% saponin (Sigma) in PBS/BSLM for 15 min at room temperature. Anti-FLAG polyclonal antibody (OctA-Probe, Santa Cruz Biotechnology) was used at 1 μg/ml in fluorescence dilution buffer (7% fetal bovine serum and 2% bovine serum albumin in PBSCM) and incubated with the coverslip for 1 h at room temperature. For single and double stains of FLAGtagged hSPRY2, fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG (Roche Molecular Chemicals) was used. For double labeling, HA-tagged c-Cbl was detected using anti-HA monoclonal antibody (Roche Molecular Chemicals) and Texas Red® dye-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) diluted in FBS. Coverslips were mounted in Crystal Mount reagent (BioMeda) and viewed by MRC-1024 laser scanning confocal microscopy (Bio-Rad). The microscopic images were processed with the aid of LaserSharp software (Bio-Rad) and Adobe Photoshop software. Mutations of the first cysteine in the Ring finger region of c-Cbl to alanine (C381A) was performed using the QuikChange mutagenesis kit (Stratagene) and polymerase chain reaction according to the manufacturer’s instruction. The mutant product was derived using wild-type c-Cbl in pXJ40HA as a template and has been verified by sequencing.

**EGFR Down-regulation Assay—**To quantify the rate of receptor in-
ternalization, Chinese hamster ovary cells were seeded at 50% density in 24-well plates. At 24 h after passage, cells were transfected with a total 1.5 µg of various expression constructs overnight before subsequent serum starvation. Cells were then incubated in fresh serum-free medium containing 0.1% of the proteasome inhibitor MG132 (N-benzyl-oxycarbonyl-Leu-Leu-Leu-aldehyde, prepared in dimethyl sulfoxide; Sigma) for 2 h. Cells were subsequently stimulated with 100 ng/ml EGF in binding buffer (RPMI 1640 medium containing 0.5% bovine serum albumin) at 37 °C for various time intervals in quadruplicates. At the end of the incubation, cells were rinsed with cold binding buffer, and unbound ligands were removed by washing three times in ligand stripping buffer (150 mM acetic acid and 150 mM NaCl (pH 2.7)). To determine the relative number of EGF receptors on the cell surface, duplicate incubations with either 10 ng/ml [125I]-EGF alone or [125I]-EGF + 100-fold excess EGF (to account for nonspecific binding) were set up and allowed to proceed for 2 h at 4 °C. After treatment, cells were rinsed with binding buffer and solubilized with 0.1% NaOH and 0.1% SDS at 37 °C for 1 h prior to counting on a γ-counter.

In Vitro Translation and Pull-down—The TNT T7 Quick Coupled transcription/translation system (Promega) was used according to the manufacturer’s protocol. The translated proteins were incubated with 10 µg of each GST fusion protein bound to glutathione-Sepharose beads at 4 °C for 4 h. Beads were collected and washed with cold radioimmune precipitation assay buffer, and the eluted proteins samples were resolved by SDS-PAGE. The gel was then dried and subjected to autoradiography.

Yeast Two-hybrid Interaction Assay—The cDNAs encoding full-length hSPRY2 (residues 1–315), hSPRY2-N (residues 1–177), and hSPRY2-C (residues 178–315) were subcloned into the pAS vector for yeast expression of Gal4 binding domain fusion proteins. Full-length c-Cbl (residues 1–906), Cbl-C (residues 437–906), and Cbl-C2 (residues 457–906) were subcloned into the pACT vector for expression of Gal4 activation domain fusion proteins. The binding domain constructs were individually transformed into Y190 yeast host, and transformants were selected on Trp-free synthetic dextrose medium plates at 30 °C for 2 days. Following yeast protein extraction and SDS-PAGE, fusion protein-expressing clones were identified by immunoblot analysis. Positive single transformants then underwent a successive round of transformation. The full-length sequences of human, mouse, and D. melanogaster SPRY (accession number AACO4257), mouse SPRY (accession number AF176903.1), and mouse SPRY4 (accession number NM011897), respectively, were amplified by PCR using the following primers: SPRY-forward (5′-CGG GGA GCC ATG GGT GGC G-3′) and SPRY-reverse (5′-GCA GGC CAG TTA GGA GGA ATG-3′). The PCR products were subcloned into the pBlueScript II KS(−) vector, and the plasmids were transformed into DH5α competent cells. Recombinant plasmids were identified by colony PCR and DNA sequencing.

In Vitro Protein Binding Assay—GST-tagged hSPRY2 fusion protein bound to glutathione-Sepharose 4B beads was eluted using 1 bed volume of 10 mM reduced glutathione (Sigma) in 50 mM Tris-HCl (pH 8.0) at room temperature for 10 min. 5-µg amounts of GST-hSPRY2 protein were incubated with 5 µg of each His-tagged c-Cbl fusion protein bound to Ni2+-nitrilotriacetic acid beads (QIAGEN GmbH) at 4 °C overnight in 500 µl of radioimmune precipitation assay buffer. Beads were collected and washed with cold radioimmune precipitation assay buffer and the eluted proteins samples were resolved by SDS-PAGE and Western-blotted with anti-Cbl antibody.

The full-length sequences of human SPRY2 (GenBank™/EBI accession number AF039843), mouse SPRY2 (accession number NM011897), Drosophila SPRY (accession number AAC04257), mouse SPRY1 (accession number AF176903.1), mouse SPRY4 (accession number AF176906.1), human Cbl (accession number X57110), and mouse Cbl-2 (accession number X92233) were aligned using the ClustalW method under DNASTAR application. Analogous Sprouty sequences spanning residues 36–53 of hSPRY2 as well as the sequences encoding SPRY1 (accession number AF176903.1), mouse SPRY4 (accession number NM011897), mouse SPRY2 (accession number AF039843), mouse SPRY2 (accession number AF039843), and mouse SPRY2 (accession number AF039843) were amplified by PCR using the following primers: SPRY-forward (5′-CGG GGA GCC ATG GGT GGC G-3′) and SPRY-reverse (5′-GCA GGC CAG TTA GGA GGA ATG-3′). The PCR products were subcloned into the pBlueScript II KS(−) vector, and the plasmids were transformed into DH5α competent cells. Recombinant plasmids were identified by colony PCR and DNA sequencing.

RESULTS

Human SPRY2 Associates Constitutively with Isoforms of Cbl—To identify binding partners of hSPRY2, we reasoned that potential binding candidates could be substrates of RTKs. With this notion in mind, we stimulated 293T cells with EGF and then exposed them to radioimmune precipitation assay buffer, and the eluted proteins samples were resolved by SDS-PAGE. The gel was then dried and subjected to autoradiography.

Western blot analysis revealed that the 190-kDa band was most likely EGFR (data not shown), and the double bands at 120 kDa correspond to c-Cbl and Cbl-b. The 55-kDa band is currently unidentified. We further observed that binding of the Cbl proteins to hSPRY2 was independent of EGF stimulation and tyrosine phosphorylation; the 120-kDa bands were equivalent in intensity in pull-down assays using both stimulated and nonstimulated cell lysates (Fig. 1A, middle and lower panels).

The results obtained indicate the existence of a complex consisting of Cbl isoforms, EGFR, hSPRY2, and the 55-kDa unidentified protein. As c-Cbl has been implicated as a down-regulator of RTK signaling and had been previously shown to bind to EGF, we wanted to characterize the nature of the interaction of c-Cbl with hSPRY2 and to explore the functional significance of their association. To confirm the interaction between hSPRY2 and Cbl in vivo, FLAG-hSPRY2 was expressed in 293T cells, and EGF-treated or untreated cell lysates were immunoprecipitated using anti-c-Cbl antibodies. As

![Image](http://www.jbc.org/Downloaded from http://www.rcr.org/)
shown in Fig. 1B (upper panel), the tyrosine-phosphorylated 190- and 120-kDa bands were present in both whole cell lysates and the anti-c-Cbl immunoprecipitates as analyzed by Western blotting with PY20. From the anti-FLAG immunoblot (Fig. 1B, middle panel), it is apparent that equal amounts of hSPRY2 were present in the c-Cbl immunoprecipitates from both nonstimulated and EGF-stimulated cells; c-Cbl therefore interacts with hSPRY2 constitutively.

The N-terminal Half of hSPRY2 Interacts Directly with the c-Cbl N-terminal Region—To demonstrate possible direct binding between hSPRY2 and c-Cbl, three different approaches were taken: binding of in vitro translated proteins, yeast two-hybrid analysis, and an in vitro protein binding study. In the first experiment, hSPRY2 was [35S]methionine-labeled by in vitro translation and assessed for its ability to bind to GST-c-Cbl, with both GST alone and GST-BNIP-2 as negative controls. As shown in Fig. 2A (upper panel), hSPRY2 bound to GST-c-Cbl, but not to GST alone or to GST-BNIP-2. In the reciprocal experiment, c-Cbl was in vitro translated and incubated with GST-hSPRY2 and GST-Grb2 (used as a positive control, as it has been previously shown to bind directly to c-Cbl (32)). Although in vitro translated c-Cbl bound to GST-hSPRY2 and GST-Grb2, it did not bind to GST alone or to GST-BNIP-2 (Fig. 2A, lower panel), further demonstrating specific and direct binding between hSPRY2 and c-Cbl. In the second experiment to verify direct binding, the yeast two-hybrid interaction assay was employed. From Fig. 2B (table), it is apparent that c-Cbl bound to full-length hSPRY2 and to the N-terminal half of hSPRY2, but not to the C-terminal half (which is highly conserved among all Sprouty proteins (21)). In the third experiment, eluted GST-tagged hSPRY2 protein was subjected to pull-down assays with His-tagged c-Cbl fusion protein beads. Bound proteins were resolved by SDS-PAGE, Western-blotted, and probed with anti-GST antibody to reveal the presence of GST-tagged hSPRY2. As shown in Fig. 2C, both His-tagged full-length c-Cbl and His-c-Cbl-NR fusion proteins bound directly in vitro to hSPRY2, but not the His-tagged vector alone or the His-c-Cbl-CO construct. We conclude that hSPRY2 and c-Cbl bind directly to each other and that the N-terminal half of hSPRY2 mediates its binding to the N-terminal region of c-Cbl.

Amino Acids 11–53 in the N-terminal Region of hSPRY2 Bind to c-Cbl—To further define the region in the N-terminal half of hSPRY2 that is involved in the interaction between hSPRY2 and c-Cbl, full-length FLAG-hSPRY2 and various N- and C-terminal deletion mutants of hSPRY2 (Fig. 3A) were expressed in 293T cells. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, and the immunoprecipitates were then analyzed for the presence of c-Cbl. As shown in Fig. 3B, all of the hSPRY2 deletion mutants bound c-Cbl, except the ΔN11 mutant (lane 2), which lacks amino acids 11–53. Therefore, the region of hSPRY2 spanning residues 11–53 is important for its interaction with c-Cbl.

c-Cbl and hSPRY2, but Not hSPRY2ΔN11, Associate in Vivo—Immunofluorescence studies were performed to investigate the cellular localizations of c-Cbl, hSPRY2, and the characterized non-binding mutant of hSPRY2 (hSPRY2ΔN11). COS-1 cells were employed for this study, as they have been previously characterized with respect to hSPRY2 localization and translocation (29). Cells were singly transfected with each construct or cotransfected with HA-c-Cbl and either the FLAG-hSPRY2 or FLAG-hSPRY2ΔN11 mutant construct. As shown in Fig. 4A, when expressed alone, c-Cbl was mostly found diffused in the cytosol (33) in both quiescent and EGF-stimulated cells (first column). As previously reported (29), singly transfected hSPRY2 demonstrates a mainly cytosolic disposition, where it aligns on microtubules in nonstimulated cells.
and translocates to membrane ruffles upon EGF treatment. When singly expressed in the quiescent state, hSPRY2 is cytosolic and does not coincide with that of hSPRY2 in the second cells (Fig. 4C, first and second cells). In the EGF-activated state, the hSPRY2 protein translocated to the membrane normally, whereas c-Cbl remained diffused in the cytosol (Fig. 4C, third and fourth cells). These results provide further evidence that amino acids 11–53 of hSPRY2 interact with c-Cbl, and this interaction can influence the spatial distribution of c-Cbl in the cell.

hSPRY2 Binds to the Ring Finger Domain of c-Cbl—The previous yeast two-hybrid analysis demonstrated that the N-terminal half of c-Cbl was responsible for binding to hSPRY2 (Fig. 2B). Experiments were then performed to further define the region of c-Cbl that affects its binding to hSPRY2. Truncation constructs of c-Cbl, depicted schematically in Fig. 5A, were subcloned into the pXJ40HA vector and tested for their ability to bind to GST-hSPRY2. 293T cells were transfected with full-length HA-c-Cbl and its truncation constructs, and cell lysates were subjected to pull-down assays with GST-hSPRY2. Bound proteins were separated by SDS-PAGE, Western-blotted, and probed with anti-HA antibody to reveal the presence of c-Cbl. As shown in Fig. 5B (lower panel), only full-length c-Cbl (denoted F in lane 2) and those truncation mutants of c-Cbl that contain an intact Ring finger domain could bind to hSPRY2 (namely Cbl-N3, lane 3, Cbl-C7, lane 8, and Cbl-C9, lane 9). These results indicate that hSPRY2 interacts with the Ring finger domain of c-Cbl.

hSPRY2, but Not hSPRY2ΔN11, Inhibits c-Cbl-mediated EGFR Down-regulation—Members of the Sla-1/Cbl and Sprouty families have been demonstrated to be involved in down-regulation of Ras/MAPK signaling by exerting their effects in close proximity to various RTKs (17, 22). Following the initial characterization of the binding between hSPRY2 and c-Cbl and their apparent colocalization in nonstimulated and RTK-stimulated cells, it was hypothesized that hSPRY2 might direct cytosolic c-Cbl to a functional intracellular location. Recently, c-Cbl has been reported to target various RTKs and non-RTKs for degradation by catalyzing the polyubiquitination of such target proteins (20, 34); more specifically, its Ring finger domain was shown to contain ubiquitin ligase activity (35). It is therefore plausible that hSPRY2 functions also to regulate the ubiquitin ligase activity of c-Cbl via its specific binding to the Ring finger domain of the latter.

An experiment was thus performed to investigate the effect of hSPRY2 on c-Cbl-induced down-regulation of stimulated EGFR. The kinetics of EGFR turnover (a measure of the rate of receptor internalization) in Chinese hamster ovary cells coexpressing EGFR with wild-type c-Cbl, c-Cbl-C81A (a dominantly negative c-Cbl Ring finger mutant), c-Cbl + hSPRY2, or c-Cbl + hSPRY2ΔN11 were quantitated according to the methodology of Waterman et al. (36). Transfectants were stimulated with EGF for various time points before pulse labeling with 125I-EGF. The amounts of 125I-EGF bound to EGFRs remaining on the cell surface after treatment were then measured. In line with the previous observation that the down-regulation of EGFR is related to an increase in c-Cbl-catalyzed polyubiquitination and subsequent destruction of the receptors (36), for cells coexpressing c-Cbl, the EGFR population remaining on the cell surface was decreased by almost 60% compared with the vector control after 30 min of EGF stimulation (Fig. 6). Furthermore, consistent with the importance of the Ring finger domain as previously observed (37), coexpression of the catalytically inactive c-Cbl-C81A mutant showed no suppressive activity. Coexpression of hSPRY2 alone also caused no significant change in the rate of EGFR internalization. However, coexpression of c-Cbl and hSPRY2 was shown to abrogate the down-regulatory effect (i.e. an elevated rate of receptor internalization) exhibited by c-Cbl alone on EGFR. Significantly, the non-binding hSPRY2ΔN11 mutant had no effect on c-Cbl-enhanced receptor turnover. As postulated, the binding of hSPRY2 to the Ring finger domain of c-Cbl inhibited the down-regulatory effect of c-Cbl on EGFR.

Drosophila Sprouty Associates with Drosophila Cbl—Evidence has been presented that mammalian SPRY2 may have a parallel function in lung development compared with Drosophila Sprouty in tracheal branching (21, 27). Therefore, it was of interest to investigate if dSPRY could similarly bind to dCbl. Although the Ring finger domain of Cbl from various species is highly conserved (Fig. 7A), the homology between the N termini of Sprouty proteins is relatively low. To assess a possible interaction between dCbl and dSPRY, HA-dCbl or HA-DRK
Fig. 4. c-Cbl and hSPRY2, but not hSPRY2an11, associate in vivo.

COS-1 cells were singly transfected with 1 μg each of HA-c-Cbl, FLAG-hSPRY2, and FLAG-hSPRY2an11 (A); with 1 μg each of HA-c-Cbl and FLAG-hSPRY2 (B); and with 1 μg each of HA-c-Cbl and FLAG-hSPRY2an11 (C). O denotes cells in a quiescent state, and E denotes EGF-stimulated cells. FLAG-tagged Sprouty constructs were stained using an anti-FLAG polyclonal antibody and fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG. HA-tagged c-Cbl was detected with an anti-HA monoclonal antibody and Texas Red® dye-conjugated AffiniPure goat anti-mouse IgG. In c-Cbl- and hSPRY2-cotransfected cells, c-Cbl colocalized with hSPRY2 in membrane ruffles (solid arrows) upon EGF stimulation (B, first and second cells; c-Cbl was found to colocalize with hSPRY2 in membrane ruffles (solid arrows) upon EGF stimulation (B, third and fourth cells). In c-Cbl- and hSPRY2an11-cotransfected cells in the quiescent state, c-Cbl appeared diffuse, but did not colocalize with hSPRY2an11 on microtubules in the cytosol (C, first and second cells). With EGF stimulation, c-Cbl remained cytosolic and did not appear with hSPRY2an11 in the membrane ruffles (C, third and fourth cells).

The binding domain to a smaller region. FLAG-tagged hSPRY2 truncation and deletion constructions as depicted in Fig. 8B were expressed in 293T cells, and cell lysates were immunoprecipitated with anti-FLAG antibody and probed with anti-c-Cbl antibody. The data shown in Fig. 8C (upper panel) indicate a lack of binding between c-Cbl and the 53C (without residues 1–53; lane 3) and ΔN36 (lacking amino acids 36–53; lane 4) mutants, whereas binding was apparent between c-Cbl and the 30C (without residues 1–30; lane 2) and full-length (lane 5) constructs. A reciprocal precipitation experiment was performed in which cell lysates from the same transfections were subjected to immunoprecipitation with anti-c-Cbl antibody and immunoblotted with anti-FLAG antibody (Fig. 8C, third panel). The result is in agreement with the above data. Thus, we further delineated that the c-Cbl-binding region of hSPRY2 is contained within sequence 36–53.

N-terminal Amino Acids 179–199 of dSPRY Bind to the Ring Finger Domain of dCbl—To extend the binding investigation to dSPRY and dCbl, various constructs as depicted in Fig. 8D were made. First, to investigate whether the Ring finger domain of dCbl was involved in binding to dSPRY, 293T cells were transiently transfected with dCbl, dCblRF, or vector alone. Cell lysates were subjected to pull-down assays with GST-dSPRY or GST alone. As shown in Fig. 8E (lower panel), whereas full-length dCbl bound to dSPRY, dCblRF (lacking the Ring finger domain) did not. This result is indicative of the Ring finger domain of dCbl being involved in its binding to dSPRY. Second, binding domain analyses were performed to ascertain the site in dSPRY that binds to the Ring finger domain of dCbl. The possible involvement of residues 179–199 in dSPRY was directly addressed. FLAG-tagged full-length dSPRY and the dSPRYn210 (amino acids 1–210), dSPRY202C (amino acids 202–592), and dSPRYΔN179 (mutant with a deletion of amino acids 179–199) constructs were transiently
c-Cbl has been reported in a number of cell systems to be tyrosine-phosphorylated upon receptor stimulation and was found to exert a negative regulatory role in tyrosine kinase signaling, albeit by an as yet undefined mechanism (31–33). The N-terminal fragment of c-Cbl essentially consists of two functional domains: an unconventional SH2-like domain (which incorporates the four-helix bundle and EF-hand) and a Ring finger motif. Structure-based mutation studies in the four-helix bundle, EF, and SH2 domains revealed that the three domains together form an integrated phosphoprotein recognition module (16). Furthermore, a critical role of the SH2 domain in c-Cbl function is demonstrated by the localization of a loss-of-function mutation in SLI-1 (C. elegans homolog of Cbl) within a 17-amino acid deletion N-terminal to the Ring finger (18); this structural alteration renders the 70Z-Cbl mutant oncogenic and causes it to exhibit an enhanced level of tyrosine phosphorylation as well as to abrogate the negative regulatory function of wild-type c-Cbl. In our binding domain analysis, we showed that neither disruption nor lack of the SH2 domain function of wild-type c-Cbl. In our binding domain analysis, we showed that neither disruption nor lack of the SH2 domain function of wild-type c-Cbl. In our binding domain analysis, we showed that neither disruption nor lack of the SH2 domain function of wild-type c-Cbl.

FIG. 6. hSPRY2, but not hSPRY2ΔN11, inhibits c-Cbl-mediated EGFR down-regulation. Chinese hamster ovary cells were transfected with 1.5 μg of total EGFR expression construct together with plasmids encoding the respective gene products (vector control (○), c-Cbl (●), Cbl-C381A (▲), hSPRY2 (■), c-Cbl and hSPRY2 (▲), or c-Cbl and hSPRY2ΔN11 (▲)) in 24-well plates. At 48 h post-transfection, duplicate wells were incubated with EGF (100 ng/ml) at 37 °C for various time intervals as indicated. Unbound EGF was then stripped off, and the levels of surface EGFR were determined by a competitive binding assay with 125I-EGF (see “Experimental Procedures”). There were an average of 70,000 cpm at the 100% EGFR level.

FIG. 5. hSPRY2 binds to the Ring finger domain of c-Cbl. A, shown is a schematic representation of HA-tagged full-length c-Cbl (FL; residues 1–906) and truncation constructs Cbl-N1 (residues 1–436), Cbl-N2 (residues 1–436), Cbl-N3 (residues 1–290) Cbl-N5 (residues 1–292), Cbl-C1 (residues 390–906), and Cbl-C2 (residues 362–906). B, four-helix bundle; EF, calcium-binding motif; PRO, proline-rich domain; LZ, leucine zipper motif. B, 293T cells were transfected (T7) with the HA-c-Cbl constructs as represented in A (where F is HA-tagged full-length c-Cbl), and lysates were subjected to pull-down (PD) assays with GST-hSPRY2. The whole cell lysate (WCL) blot was probed with anti-HA antibody to show expression levels of the various proteins (upper panel). Bound proteins were immunoblotted (IB) with anti-HA antibody to check for binding of the various forms of c-Cbl to GST-hSPRY2 (lower panel). O, vector alone.

DISCUSSION

Recently, the Cbl family proteins have attracted a considerable amount of attention as down-regulators of both RTK and non-RTK signaling (34, 39). Genetic analysis implicated the orthologs of Cbl in C. elegans and Drosophila in down-regulating the EGFR growth-promoting function (17, 20, 38). Human

expressed in 293T cells, and lysates were incubated with either GST alone or GST-dCbl. The binding data shown in Fig. 8F (lower panel) indicate that dSPRY-derived proteins that contain residues 179–199 bound to dCbl, whereas those that lack the sequence did not. The region comprising residues 179–199 of dSPRY is therefore responsible for its interaction with dCbl; deletion of this region of dSPRY can similarly abolish its binding to c-Cbl (data not shown).
case, tyrosine residues on activated EGFR become phosphorylated upon growth factor stimulation and bind to the SH2 domain of c-Cbl; c-Cbl functions as an E3 to mediate endocytic sorting of the target substrate by relaying activated ubiquitin molecules to EGFR via its Ring finger. Although all three members of the Cbl family (c-Cbl, Cbl-b, and Cbl-3) can enhance ubiquitination (40), two oncogenic variants (70Z-Cbl and v-Cbl) whose Ring fingers are defective are unable to desensitize EGFR (18, 20). The oncogenic viral counterpart (v-Cbl), which lacks a functional Ring finger, inhibits down-regulation by shunting endocytosed receptors to the recycling pathway. This has exciting implications because, in relation to our findings, binding of hSPRY2 to the catalytic site of c-Cbl would suggest an important modulatory role of hSPRY2 in the fate and signaling potency of growth factor receptors. Additionally, we found that hSPRY2 binding to the Ring finger domain of c-Cbl abrogates the latter’s ability to induce down-regulation of EGFR.

Our studies have demonstrated that hSPRY2 binds directly to the Ring finger domain of c-Cbl (and possibly also Cbl-b) via a small N-terminal region. A similar observation was made for dSPRY and dCbl, the binding of which encompasses similar regions. This is indicative of a conservation of the interaction domains throughout evolution. In this respect, it is interesting to note that there is no Sprouty ortholog found in C. elegans (347x291).

Taken together, the constitutive nature of the hSPRY2-c-Cbl association may highlight the requirement for some extrinsic factors to displace hSPRY2 from the Ring finger domain of c-Cbl and, by so doing, uplifts its suppressive effect on the latter.

A number of questions are posed by the data presented. First of all, do the other mammalian Sprouty proteins also bind to the Ring finger of c-Cbl? Preliminary analysis revealed that murine SPRY1 binds c-Cbl, but not murine SPRY4 (data not shown). Hence, one might infer that mSPRY4 plays a different functional role from mSPRY1 and mSPRY2. We have recently presented evidence that the conserved cysteine-rich C-terminal domain in various Sprouty proteins is responsible for directing the proteins to membrane ruffles when cells are stimulated...
with growth factors (29). Given the high degree of conservation among the C-terminal regions, this would suggest that Sprouty proteins have a common target for membrane ruffle association. We further demonstrated that c-Cbl colocalizes with hSPRY2 in coexpressing cells, but not with the non-binding hSPRY2ΔN11 mutant. It becomes interesting then to question

**Fig. 8.** Common SPRY Cbl-binding regions in mammalian and Drosophila systems. A, shown is an amino acid sequence alignment of the putative Cbl-binding regions of various Sprouty orthologs. Identical residues are indicated in black. Dashes indicate no corresponding amino acid at that position. B, shown is a schematic representation of FLAG-tagged hSPRY2 constructs to determine whether amino acids 36–53 are responsible for binding to c-Cbl. Shown are the full-length (FL; residues 1–315), 230C (residues 30–315), 53C (residues 53–315), and ΔN36 (with a deletion of residues 36–53) hSPRY2 fragments. C, 293T cells were transfected (Tf) with the FLAG-tagged hSPRY2 constructs represented in B. The upper panel shows the presence of endogenous c-Cbl bound by immunoprecipitated (IP) FLAG-hSPRY2 constructs. Precipitated amounts of FLAG-tagged proteins are shown in the second panel. For reciprocal binding, immunoprecipitated c-Cbl proteins (amounts shown in the lower panel) were checked for their binding to various FLAG-hSPRY2 constructs by immunoblotting (IB) with anti-FLAG antibody (third panel). O, vector alone. D, shown is a schematic representation of full-length dCbl (residues 1–592) and dCblΔRF (residues 1–369) and various FLAG-tagged dSPRY constructs to determine the region of binding between dCbl and dSPRY. 4H, four-helix bundle; EF, calcium-binding motif; PRO, proline-rich domain; LZ, leucine zipper motif. Shown are the full-length (SPRY-FL; residues 1–592), N210 (residues 1–210), 202C (residues 202–592), and ΔN179 (with a deletion of residues 179–199) SPRY fragments. E, dSPRY binds to the Ring finger domain of dCbl. HA-dCbl and HA-dCblΔRF were transiently expressed in 293T cells. A whole cell lysate (WCL) blot immunoblotted with anti-HA antibody shows equal protein expression (upper panel). Precipitated proteins from lysates subjected to pull-down (PD) assays with GST-dSPRY and GST alone were probed with anti-HA antibody (lower panel). F, the N-terminal amino acids 179–199 of dSPRY are responsible for binding to dCbl. FLAG-dSPRY constructs as represented in D were transiently expressed in 293T cells, and lysates were subjected to pull-down assays with GST-dCbl and GST alone. Immunoblots of whole cell lysates (upper panel) and bound proteins (lower panel) were immunoblotted with anti-FLAG antibody.
the roles of hSPRY2 and c-Cbl in each of the two localities. Does hSPRY2 translocate to ruffles to assume a different function by virtue of different or additional binding partners, or does the hSPRY2-mediated translocation serve to target c-Cbl in near proximity to the membrane-anchored receptors? If the latter case is true, one would expect hSPRY2 to synergistically promote the action of c-Cbl in the ligand-induced down-regulation of EGFR, which differs from our experimental observation. Although we do not know the proportion of endogenous c-Cbl that is bound by hSPRY2 (and therefore “nonfunctional” in terms of EGFR down-regulation), our results suggest that the inhibition of EGFR internalization that we observed in c-Cbl- and hSPRY2-coexpressing cells may be due to an exclusion of prospective E2-ubiquitin complexes from the Ring finger domain of c-Cbl, thereby preventing downstream ubiquitination events. Alternatively, hSPRY2 may have a direct influence in receptor endocytosis.

On the other hand, we cannot rule out the possibility that a fraction of the c-Cbl population that hSPRY2 binds to is tyrosine-phosphorylated or not or that, upon growth factor stimulation, hSPRY2 initiates a stronger preference for binding to other protein partners that might mediate translocation of the hSPRY2-c-Cbl complex to ruffles. It is interesting to note from the immunofluorescence studies on hSPRY2- and c-Cbl-coexpressing cells that a significant pool of c-Cbl still retains an association with microtubules in the cytosol, although a major portion has been shown to colocalize with hSPRY2 at the membrane ruffles in EGF-treated cells. Given the previously reported function of dSPRY and mSPRY2 in the attenuation of receptor endocytosis (21), it is possible that hSPRY2 associates with other signaling proteins to down-regulate the MAPK cascade. It will be interesting to pursue any substantial attenuation of MAPK signaling in hSPRY2- and c-Cbl-coexpressing cells. It is noteworthy that other studies done by overexpressing two forms of c-Cbl mutants (namely C381A and 70Z-Cbl) showed an inhibitory effect on EGFR down-regulation (18, 37).

Do the various Sprouty isoforms bind to the relatively well conserved Ring finger domains of proteins other than c-Cbl? Despite the increasing number of proteins known to be ubiquitinated, the identification of the corresponding ubiquitin ligases (E3) has been lagging. E3 ligases for which the amino acid sequences are known include the N-end rule E3 ligases of the APC, the anaphase-promoting complexes (49), Ring finger motifs intrinsic to a large number of proteins have sur-

REFERENCES

1. Marshall, C. J. (1995) Cell 80, 179–185
2. Porter, A. C., and Vaillancourt, R. R. (1998) Oncogene 17, R1343–R1352
3. Tan, P. B., and Kim, S. K. (1999) Trends Genet. 15, 145–149
4. Marshall, C. J. (1994) Curr. Opin. Cell Biol. 6, 1175–1188
5. Campbell, S. L., Khoarev-Farr, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998) Oncogene 17, R1381–R1413
6. Kaykas, S. M. (2000) Curr. Opin. Cell Biol. 12, 186–192
7. Pltsnikov, A. N., Schlessinger, J., Hubbard, S. R., and Mohammadi, M. (1999) Cell 98, 641–650
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