Tesk1 Interacts with Spry2 to Abrogate Its Inhibition of ERK Phosphorylation Downstream of Receptor Tyrosine Kinase Signaling

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The Sprouty (Spry) proteins function as inhibitors of the Ras/ERK pathway downstream of various receptor tyrosine kinases. In this study, we have identified Tesk1 (testicular protein kinase 1) as a novel regulator of Spry2 function. Endogenous Tesk1 and Spry2 exist in a complex in cell lines and mouse tissues. Tesk1 coexpression relocalizes Spry2 to vesicles including endosomes, inhibiting its translocation to membrane ruffles upon growth factor stimulation. Independent of its kinase activity, Tesk1 binding leads to a loss of Spry2 function as an inhibitor of ERK phosphorylation and reverses inhibition of basic fibroblast growth factor (bFGF)- and nerve growth factor-induced neurite outgrowth in PC12 cells by Spry2. Furthermore, depletion of endogenous Tesk1 in PC12 cells leads to a reduction in neurite outgrowth induced by bFGF. Tesk1 nullifies the inhibitory effect of Spry2 by abrogating its interaction with the adaptor protein Grb2 and interfering with its serine dephosphorylation upon bFGF and FGF receptor 1 stimulation by impeding its binding to the catalytic subunit of protein phosphatase 2A. A construct of Tesk1 that binds to Spry2 but does not localize to the vesicles does not interfere with its function, highlighting the importance of subcellular localization of Tesk1 in this context. Conversely, Tesk1 does not affect interaction of Spry2 with the E3 ubiquitin ligase, c-Cbl, and consequently, does not affect its inhibition of Cbl-mediated ubiquitination of the epidermal growth factor receptor. By selectively modulating the downstream effects of Spry2, Tesk1 may thus serve as a molecular determinant of the signaling outcome.

Sprouty (Spry) was first discovered in Drosophila as a negative feedback regulator of receptor tyrosine kinase signaling (1, 2), a function that is seemingly conserved in its four mammalian homologs, Spry1–4 (3, 4). Although the Spry proteins contain no known domains, they all have a unique, highly conserved Cys-rich C-terminal region. This sequence was not known to exist in any other protein until the discovery of Spred (Sprouty-related proteins with EVH1 domain) (5), of which three mammalian isoforms have been currently identified. Various isoforms of Spry and Spred are reported to be deregulated in cancers, including those of the breast, prostate, and liver (reviewed in Ref. 6), suggesting a potential role for them as tumor suppressors. A recent report that Spry2 regulates oncogenic K-Ras signaling (7) has added strength to this argument. Understanding their regulation and mechanism of action is thus a necessary step toward validating these proteins as therapeutic targets for disease treatment.

Spry and Spred share several features, including extensive homology over their Cys-rich C terminus (8). The best characterized function for both protein families is as inhibitors of ERK3 phosphorylation downstream of a range of stimuli including growth factors and cytokines. However, their similarity does not seem to extend to their mode of action. Although various groups have reported the point of action of Spry as being either upstream of Ras by disrupting the Grb2-Sos complex (9) or at the level of Raf activation (10, 11), the current understanding of Spry function suggests that it may interfere with Ras-induced activation of Raf (5).

To date, a number of binding partners to Spry have been discovered including c-Cbl, Grb2, Raf-1, Caveolin1, Tesk1 (testicular protein kinase 1), PTP1B, and PTEN (2, 11–16). Of these, Raf-1 and Caveolin1 are also reported to bind to Spry2 (16) and are deemed necessary for inhibition of ERK phosphorylation by both Spry and Spred.

Tesk1 belongs to a novel family of coflin kinases comprising of two members (Tesk1 and 2) that share similarity with the LIM (Lin-11, Isl-1, and Mec-3) kinases in the catalytic domain (18). Tesk1 regulates actin dynamics in the context of cell adhesion and spreading downstream of integrin signaling by phosphorylating coflin on the Ser residue at position 3, thereby inactivating it. Spry4 has previously been reported to bind to Tesk1 and negatively regulate its kinase activity, resulting in inhibition of cell spreading (14, 19). In this study, we present

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evidence that Tesk1, in turn, is a novel regulator of Spry2 that differentially modulates its downstream effects.

EXPERIMENTAL PROCEDURES

Plasmids—Rat Tesk1 and Tesk2 full-length constructs in pCAG-Myc vector have been described earlier (18). The D170A and truncation Tr528 mutants were generated by site-directed mutagenesis using the proof-reading Pfu DNA polymerase (Promega, Madison, WI). FGFRI, HA-c-Cbl, FLAG-tagged Spry1, Spry2, Spry4, and ERK2 have been described earlier (9, 10, 12). Full-length Spred1 and 2 amplified from mouse brain cDNA and Spry3 amplified from MCF-7 cDNA were cloned into pXJ40-FLAG mammalian expression vector (20). The C100 construct was generated by subcloning the yeast two-hybrid fragment into pXJ40-Myc. pXJ40-FLAG-Spry2-N and -C, pXJ40-HA-Ubiquitin, and EGFP-Rab7 were kind gifts from Chow Soah Yee, Dr. Fu Naiyang, and Dr. Zhou Zhihong.

Yeast Two-hybrid Assay—Yeast two-hybrid was performed using the Match Maker III system from Clontech (Mountain View, CA) following the manufacturer’s instructions. Full-length Spred1 was subcloned into pGBK-T7 vector and used as bait on a pretransformed mouse brain library cloned into pACT2 vector. Positive clones were selected on triple dropout medium (lacking His/Leu/Trp) and further confirmed on high stringency quadruple dropout (lacking His/Leu/Trp/Ade). Plasmids were isolated, transformed into Escherichia coli DH10B and sequenced to identify and determine the reading frame of the clones. Open reading frames of positive in-frame clones were cloned into pXJ40 vector for expression in mammalian cells.

Antibodies and Reagents—Mouse (M2) and rabbit anti-FLAG, rabbit anti-Spry2 (N-terminal), anti-NA, and Cy3-conjugated mouse anti-tubulin were from Sigma-Aldrich. Mouse monoclonal anti-Myc (9E10), anti-EGFR (R1), and rabbit antibodies against FGFRI, EGFR, Myc, and Grb2 were from Santa Cruz Biotechnology (Santa Cruz, CA). EGF was from Upstate Biotechnology Inc. (Lake Placid, NY). Mouse monoclonal anti-Tesk1 (clone 1D11) was from Novus Biologicals (Taiwan). Mouse antibodies against Grb2, GM130, EA1, Rab5, and pan ERK were from BD Transduction Laboratories (Lexington, KY). Mouse anti-phospho ERK1/2 and anti-PP2A-C were from Cell Signaling Technology (Beverly, MA). Mouse anti-LAMP1 was from Abcam. Alexa Fluor 488 donkey anti-mouse IgG and Alexa Fluor 647 goat anti-rabbit IgG were from Molecular Probes Inc. (Eugene, OR).

Cell Lines and Transfection—All cell lines used in this study were purchased from ATCC (Manassas, VA). HEK293T, Cos1, and PC12 cells were cultured and maintained as described previously (21). All cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were performed essentially as described (9). For the endogenous immunoprecipitation of cell lines and mouse tissue lysates, the samples were lysed in a HEPES-based lysis buffer (10) and rotated at 4 °C for 1 h to ensure complete lysis. After extensive centrifugation, 5 mg (cell lines) or 10 mg (mouse tissue) of total protein was subjected to immunoprecipitation with 10 μg of rabbit anti-Spry2 (N-terminal) antibody or normal rabbit immunoglobulin G (IgG) as control. The resulting immunoprecipitates were separated on SDS-PAGE and blotted with mouse anti-Tesk1 and rabbit anti-Spry2 (N-terminal) to detect the proteins.

PC12 Neurite Outgrowth Assay—PC12 cells were plated on poly-L-lysine-coated coverslips and transfected with the various constructs indicated. 24 h post transfection, the cells were quiesced in medium containing 0.2% fetal bovine serum and subsequently stimulated with 50 ng/ml basic fibroblast growth factor for 4 days or nerve growth factor for 2 days. The cells were fixed and visualized as described below.

Immunofluorescence Microscopy—Cos1 and PC12 cells were fixed, stained, and visualized essentially as described (21) with the following modifications. Permeabilized cells were blocked overnight with phosphate-buffered saline supplemented with 1 mM each of CaCl₂ and MgCl₂, 2% bovine serum albumin, and 7% fetal bovine serum. Primary and secondary antibodies were diluted in the same solution and incubated at room temperature for 1 h each. The images were captured using a Zeiss LSM 510 META (Carl Zeiss Microimaging).

Short Hairpin RNA Knockdown—Short hairpin RNA (shRNA) constructs against Tesk1 (catalog number TR308879) were purchased from Origene Technologies, Inc. The targeted sequences were: AAGTCATGTTGCTGAAATGAAACAA-GTGC (#9), TTGGCCTGATGTCGGCTTTCCGACT (#10), CAGACACATTCGACCGTCCCT (#11), and AAGCAGTCTGCTCTTGTGCCACCATC (#12). The shRNAs containing the shRNA were transduced into 293T and PC12 cells using normal transfection methods described above. For PC12 cells, equal amounts of EGFP and shRNA plasmids were cotransfected in each experiment.

RESULTS

Tesk1 Is a Common Interacting Partner to Spred and Spry—With the view that the nature of associated proteins will provide clues to the physiological function of newly discovered proteins such as Spred, we performed a yeast two-hybrid screen using an adult mouse brain library with full-length Spred1 as bait. One of the positive clones recovered from the screen encoded for the last 100 amino acid residues of Tesk1 (designated C100, Fig. 1A). When expressed in HEK293T cells, this fragment of Tesk1 coimmunoprecipitates with full-length Spred1 (Fig. 1B). Among the different regions of Spred1 (schematically shown in Fig. 1A), namely the EVH1 (residues 1–136), the EVH2 (residues 137–332), and the Cys-rich region (C terminus; residues 333–444), C100 interacts strongly with the Cys-rich C terminus of Spred1 (Fig. 1C).

Since the Cys-rich C terminus of Spred is conserved with that of the Spry proteins and a previous study reported that Spry4 interacts with Tesk1 through this region (19), we sought to verify whether Tesk1 may be a common interacting partner to both of these protein families. Furthermore, as only the C-terminal fragment of Tesk1 was isolated in the screen, we wanted to validate this interaction in the context of the full-length protein. When coexpressed, all isoforms of Spry and Spred bind to full-length Tesk1 (Fig. 2A). A mutant of Tesk1 where the catalytic aspartate at position 170 is mutated to alanine (D170A) has been shown to lack kinase activity (22). We used this mutant
(designated DA) and a truncation mutant where the last 100 residues equivalent to C100 were deleted (designated Tr528) to check whether (a) the kinase activity of Tesk1 might influence its binding to Spred and Spry and (b) the binding is mediated by the C-terminal tail of Tesk1. Although the DA mutant binds to Spred1 and Spry2 similar to WT Tesk1, Tr528 does not bind
**Tesk1 Reverses Inhibition of ERK by Spry2**

(Fig. 2B). Spry4 also showed a similar pattern of binding (data not shown). Thus, the binding seems to be mediated solely through the C-terminal 100 residues of Tesk1, independent of its kinase activity.

Tesk2 is a closely related kinase to Tesk1 that shares about 70% similarity in the kinase domain (23). The rest of their sequences have little similarity to each other except for three conserved regions designated CR1–3 (Fig. 1A). If the C terminus of Tesk1 mediated binding to Spry/Spred, it would be expected that Tesk2 would not bind as this region is not conserved between the two isoforms. This is indeed the case since none of the Spry or Spred isoforms binds to full-length Tesk2 (supplemental Fig. S1).

Tesk1 is expressed at relatively high levels in testes and at lower levels in most other tissues and cell lines (18, 24). To determine whether Tesk1 interacts endogenously with Spry and Spred, we immunoprecipitated endogenous Spry2 from lysates of cell lines and mouse tissue and assayed for the presence of Tesk1 in the immunoprecipitates. Endogenous Tesk1 coimmunoprecipitates with Spry2 in mouse testes and brain tissue, indicating that these proteins form a complex in vivo. A complex was also detected in CHO-K1 cells but not in 293T cells (Fig. 2C). The other isoforms of Spry and Spred could not be tested due to a lack of specific antibodies. To study the downstream effects of Tesk1 interaction, we used Spry2 in subsequent experiments as the result in Fig. 2C provided evidence of an endogenous complex with this isoform in addition to its being the best characterized and reportedly the strongest inhibitor of ERK phosphorylation (9, 10).

**Tesk1 Alters the Subcellular Localization of Spry2**—To determine whether Spry2 colocalized with Tesk1 in cells, we used indirect immunofluorescence to visualize their localization in Cos1 cells. Spry2 on its own localizes to microtubules and translocates to membrane ruffles upon EGF stimulation (Fig. 3A), as reported earlier (13, 25, 26). On the other hand, full-length Tesk1 WT and DA localize to vesicles irrespective of stimulation. Interestingly, the Tr528 mutant no longer localizes to the vesicles, suggesting that the C-terminal tail of Tesk1 may play a role in its own subcellular localization. The vesicular localization of Tesk1 has been previously reported (19), but the identity of these vesicles has not been determined. To this end, we costained the cells with markers for various subcellular compartments. A subset of the Tesk1-positive vesicles overlaps with the early endosomal markers EEA1 and Rab5 and the late endosomal marker Rab7 but not the Golgi marker GM130 or the lysosomal marker LAMP1 (Fig. 3B). There was also no colocalization with the endoplasmic reticulum marker calreticulin or the mitochondrial marker cytochrome c (data not shown). We noted that a subpopulation of Tesk1 vesicles did not overlap with any of the markers used in this study, the identity of which remains to be determined.

When coexpressed, Spry2 localizes to the vesicles with Tesk1 WT, and its translocation to membrane ruffles upon EGF stimulation is greatly reduced (Fig. 4, panels a and b). The DA mutant of Tesk1 has a similar effect to the WT, whereas the Tr528 does not affect Spry2 localization (panels c–f), indicating that this effect of Tesk1 is mediated by its binding rather than its kinase activity.

The membrane translocation of Spry2 is known to be mediated by its Cys-rich C terminus (21, 25). A fragment of Spry2 lacking this region (Spry2-N; residues 1–164) exhibits a cytoplasmic distribution both in the basal and the EGF-stimulated state in contrast to the Cys-rich region (Spry2-C; residues 165–315), which shows a clear translocation to the membrane upon

![Figure 3](image-url)
Although the subcellular distribution of Spry2-N is not altered in the presence of Tesk1 (Fig. 5C, panels a and b), the change in the localization of Spry2-C reflects that of the full-length both in the basal and stimulated states (panels c and d). This is also in agreement with the earlier observation that the homologous region of Spred1 mediates the binding to Tesk1 (Fig. 1C).

Tesk1 Negatively Regulates the Role of Spry2 as an Inhibitor of the Ras/ERK Pathway—One of the best known functions of Spry2 is as an inhibitor of the Ras/ERK pathway (8). We used two different systems to determine whether its interaction with Tesk1 had any influence on this function. In the first, cells cotransfected with Spry2 and Tesk1 were stimulated with bFGF, and the levels of phosphorylated ERK were assayed. As seen in Fig. 6A, although Spry2 on its own inhibits ERK phosphorylation compared with control levels (lanes 5 and 7), coexpression of Tesk1 reverses this effect (lane 8), whereas Tesk1 itself does not significantly affect the levels of phosphorylated ERK on its own. We further verified this effect of Tesk1 using overexpression of FGFR1, which is auto-activating, to reflect long term signaling as seen during oncogenesis and development. In this system as well, Tesk1 reverses the inhibition of ERK phosphorylation by Spry2 (Fig. 6B, lanes 8 and 9). It appears that the kinase activity of Tesk1 does not play a role in this effect, since the kinase dead DA mutant has a similar effect to the WT (Fig. 6C, lane 15), whereas the Tr528 construct that retains its kinase activity, but no longer binds to Spry2, does not influence its function (Fig. 6C, lane 17).

Tesk1 Rescues Neurite Outgrowth Inhibited by Spry2 in PC12 Cells—One of the widely studied downstream consequences of the Ras/ERK inhibitory effect of Spry2 is its inhibition of bFGF-induced neurite outgrowth in the rat pheochromocytoma cells, PC12 (9, 27–29). To establish whether the reversal of its ERK inhibition by Tesk1 translated to a cellular context, we studied the effect of Tesk1 and Spry2 coexpression in PC12 cells. As previous reports in the literature have highlighted the tight regulation on cofilin phosphorylation and dephosphorylation in neurite outgrowth (30, 31), we wanted to avoid interference from the cofilin kinase activity of Tesk1 in this assay. To this end, we used the kinase dead DA mutant of Tesk1 because it still binds to Spry2 and reverses its inhibition of ERK phosphorylation similar to WT Tesk1. When expressed in PC12 cells, Spry2 almost completely abrogates bFGF-induced neurite outgrowth (Fig. 7A, panel a). Although Tesk1 DA does not affect neurite outgrowth on its own under the same conditions (panel b), it restores neurite outgrowth in these cells in the presence of Spry2 (panel c). As further evidence, we also studied the differentiation of PC12 cells in response to NGF. The result in Fig. 7B shows that similar to the case with bFGF, Spry2 also inhibits neurite outgrowth induced by NGF (panel a), which is reversed by coexpression of Tesk1 DA (panel c).

If Tesk1 indeed antagonizes the inhibitory effect of Spry2, it follows that endogenous Tesk1 may play a positive role in this process. To determine whether this is the case, we used shRNA to knock down endogenous Tesk1 in PC12 cells. Because the Myc-Tesk1 used in this study is the rat isoform, we first tested the silencing efficiency of the shRNAs against this construct before introducing them into PC12 cells. Of those tested, shRNA#9 was the most effective in decreasing the expression of staining (Fig. 5A). As reported previously, neither the N nor the C terminus of Spry2 alone exhibits the microtubular localization seen with the full-length protein (25). After confirming that Spry2 binds to Tesk1 through its C terminus in an immunoprecipitation experiment (Fig. 5B), we determined the effect of Tesk1 coexpression on the localization of these constructs.

FIGURE 4. Tesk1 alters the subcellular localization of Spry2. Cos1 cells were cotransfected with FLAG-Spry2 and Myc-Tesk1 WT, DA, or Tr528. 24 h post-transfection cells were serum-starved for 3 h and either left unstimulated (panels a, c, and e) or stimulated with 50 ng/ml EGF (panels b, d, and f) for 30 min. The cells were fixed and stained with mouse anti-FLAG and rabbit anti-Myc antibodies followed by anti-mouse IgG (green) and anti-rabbit IgG (red). WT, wild type Tesk1; DA, kinase dead Tesk1 D170A; Tr528, truncation mutant of Tesk1 from residues 1–528. Bar, 20 μm.
The kinase activity of Tesk1 does not play a role in this effect as kinase dead Tesk1 DA has the same effect, whereas Tr528 does not (Fig. 8C, lanes 14 and 16). This result, while ruling out the possibility that Tesk1 directly phosphorylates Spry2, suggests that it involves the interaction between the two proteins.

PP2A is a multi-subunit Ser/threonine (Thr) phosphatase that has been implicated in the dephosphorylation of Spry2 (32, 33). In the latter study we have further shown that both the regulatory A and the catalytic C subunits of PP2A bind to Spry2, with the A subunit binding directly to the 50–60 region. To determine whether Tesk1 inhibited Spry2 dephosphorylation by interfering with its interaction with PP2A, we analyzed the binding of PP2A-A and -C subunits to Spry2 upon Tesk1 coexpression (Fig. 8D). Although PP2A-A binding to Spry2 is not affected, the binding of PP2A-C is greatly reduced in the presence of Tesk1, both in the basal and the FGFR1-stimulated conditions. Lack of the catalytic C subunit in the complex would thus abolish the dephosphorylating activity of PP2A on its substrate Spry2.

Vesicular Localization Is Essential for the Negative Effect of Tesk1—The negative effect of Tesk1 could potentially arise from two different scenarios. It could be due to steric hindrance such that it blocks the PP2A-C from accessing the A subunit binding directly to the 50–60 region. To determine whether Tesk1 inhibited Spry2 dephosphorylation by interfering with its interaction with PP2A, we analyzed the binding of PP2A-A and -C subunits to Spry2 upon Tesk1 coexpression (Fig. 8D). Although PP2A-A binding to Spry2 is not affected, the binding of PP2A-C is greatly reduced in the presence of Tesk1, both in the basal and the FGFR1-stimulated conditions. Lack of the catalytic C subunit in the complex would thus abolish the dephosphorylating activity of PP2A on its substrate Spry2.

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vesicles. Our results with Tr528 showed that Tesk1 binding to Spry2 and its own localization were dictated by its last 100 residues (C100). Indirect immunofluorescence, however, revealed that C100 does not by itself localize to vesicles and instead has a cytosolic and membrane distribution (Fig. 9A, panels a and b). Thus, C100 provided us with a tool to study the contribution of binding versus localization in the negative effect of Tesk1 on Spry2 function.

Spry2 colocalizes with C100 both in unstimulated and EGF-stimulated conditions when expressed in Cos1 cells (Fig. 9A, panels c and d). We noted that a subpopulation of Spry2 localizes to the plasma membrane in the presence of C100 even in

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**FIGURE 6.** Tesk1 negatively regulates the role of Spry2 as an inhibitor of ERK phosphorylation. A, 293T cells were transfected with FLAG-Spry2 and Myc-Tesk1 WT as indicated. 24 h post transfection, the cells were serum-starved for 3 h and either left unstimulated or stimulated with bFGF for 2 h. The lysates were separated on SDS-PAGE and immunoblotted (IB) with antibodies against phospho-ERK (pERK), pan-ERK, Myc, and FLAG as indicated (upper panel). B, 293T cells were transfected with Myc-Tesk1 WT, FLAG-Spry2, and FLAG-ERK2 in the presence or absence of FGFR1, as indicated. Total cell lysates (TCL) were separated on SDS-PAGE and immunoblotted with antibodies against phospho-ERK (pERK), pan-ERK, Myc, and FGFR1 (upper panel). C, 293T cells were transfected with the indicated constructs of Myc-Tesk1, FLAG-Spry2, FLAG-ERK2, and FGFR1. The lysates were separated on SDS-PAGE and immunoblotted with the antibodies as described in B. WT, wild type; DA, kinase dead Tesk1 D170A; Tr528, truncation mutant of Tesk1 from residues 1–528. A black arrowhead indicates the FLAG-ERK2 band. Graphical representation of the average of at least three independent experiments is shown in panels to the right for A–C. Cont, control.
FIGURE 7. Tesk1 rescues PC12 neurite outgrowth inhibited by Spry2. A and B, PC12 cells transfected with combinations of FLAG-Spry2 and Myc-Tesk1 DA were stimulated with 50 ng/ml bFGF for 4 days (A) or NGF for 2 days (B), fixed, and stained with mouse anti-FLAG (green), rabbit anti-Myc (red), or anti-tubulin (orange), as indicated. The arrows indicate transfected cells. C, lysates of 293T cells cotransfected with Myc-Tesk1 and four different shRNA constructs were subjected to immunoblotting (IB) with anti-Myc antibody to analyze the extent of expression knockdown. The blot was also probed with a pan-ERK antibody to ensure equal loading of lysates. D, PC12 cells cotransfected with EGFP and Tesk1 shRNA #9 (a and b) or #12 (c and d) were stimulated with bFGF for 4 days, fixed, and visualized for neurite outgrowth. The average percentage of transfected cells bearing neurites from a minimum of three independent experiments is graphically shown in the panels to the right for A, B and D. Bar, 20 μm. TCL, total cell lysates.
the basal state. We next analyzed the effect of C100 on Spry2 in an experimental set-up similar to that described in Fig. 4. C100 does not affect inhibition of ERK phosphorylation by Spry2, as opposed to the full-length WT Tesk1 (Fig. 9B, lanes 11 and 13), even though it coimmunoprecipitates with Spry2 similar to the WT (Fig. 9C). C100 also does not inhibit the dephosphorylation of Spry2 induced by FGFR1 (Fig. 9B, lane 13). It is interesting to note that in the presence of C100, Spry2 seems to be mainly concentrated in the faster migrating form even in the unstimulated state (lane 7), suggesting that it may influence the basal Ser phosphorylation of Spry2. In PC12 cells, Spry2 inhibits bFGF-induced neurite outgrowth even in the presence of C100 (Fig. 9D), further validating the fact that binding of C100 does not interfere with Spry2 function. Taken together, these results show that the negative effect of Tesk1 binding on Spry2 is more likely a case of sequestration that requires its vesicular localization.

**Tesk1 Does Not Affect c-Cbl Binding or Inhibition of EGFR Ubiquitination by Spry2**—In a previous study we have reported the presence of at least two mutually exclusive pools of Spry2: one bound to PP2A that contributes to its Ras/ERK inhibitory function and another bound to the E3 ubiquitin ligase, c-Cbl (32). The interaction between Spry2 and c-Cbl has been extensively studied (12, 34, 35) and involves the binding of the SH2-like domain of c-Cbl to a conserved tyrosine at position 55 on Spry2 (35–37). To investigate the effect of Tesk1 on the c-Cbl-associated pool of Spry2, we detected the amount of c-Cbl immunoprecipitated with Spry2 in the presence and absence of Tesk1. The result in Fig. 10A shows that Tesk1 coexpression only marginally decreases the binding of c-Cbl to Spry2. Spry2
FIGURE 9. Vesicular localization is essential for the negative effect of Tesk1 on Spry2 function. A, Cos1 cells were transfected with Myc-Tesk1 C100 alone or in combination with FLAG-Spry2, serum-starved for 3 h, and either left unstimulated (panels a and c) or stimulated with 50 ng/ml EGF for 30 min (panels b and d). The cells were fixed and stained with mouse anti-FLAG and rabbit anti-Myc, followed by anti-mouse (green) and anti-rabbit (red) IgG.

B, 293T cells were transfected with Myc-Tesk1 WT or C100, FLAG-Spry2, and FLAG-ERK2 in the presence or absence of FGFR1, as indicated. Total cell lysates (TCL) were immunoblotted (IB) with anti-phospho-ERK (pERK), pan-ERK, Myc, FLAG, and FGFR1. A black arrowhead indicates the FLAG-ERK2 band. Graphical representation of the average of at least three independent experiments is shown in the bottom panel. C, lysates from B were immunoprecipitated (IP) with anti-Spry2, resolved on SDS-PAGE, and immunoblotted with antibodies against Myc and FLAG. D, PC12 cells transfected with C100 alone or in combination with FLAG-Spry2 were stimulated with bFGF for 4 days, fixed, and stained with mouse anti-FLAG (green), rabbit anti-Myc (red), and anti-tubulin, (orange). The arrows indicate transfected cells. Bar, 20 μm.
Several groups have reported the inhibitory role of Spry2 in receptor internalization and proteasomal degradation. c-Cbl binds to and ubiquitinates EGFR upon ligand stimulation, leading to receptor internalization by Spry2 (reviewed in Ref. 38). c-Cbl-mediated ubiquitination and subsequent trafficking of EGFR (36, 39, 40) as it competes with EGFR for binding to c-Cbl via its SH2 domain. This results in increased surface retention of the receptor upon ligand binding, and consequently, prolonged downstream signaling. Because there was a slight decrease in the amount of c-Cbl bound to Spry2, our next step was to determine whether this might reflect on the ability of Spry2 to inhibit the ubiquitination of EGFR. The result in Fig. 10B shows that while EGFR gets ubiquitinated in the presence of c-Cbl upon EGF stimulation, this is reduced by coexpression of Spry2. Spry2 itself gets degraded upon EGF stimulation, as can be seen from the reduced levels of the protein, likely because of its own ubiquitination by c-Cbl. Tesk1 coexpression does not alter this pattern (lanes 7–10), suggesting that it does not significantly affect this aspect of Spry2 cellular function.

**DISCUSSION**

The identification of various interacting partners to Spry2 in recent years has increased our understanding of the intricate mechanism controlling its activation and mode of action. In this study, we report the identification of Tesk1 as a novel regulator of Spry2. That Spry2 and Tesk1 form a complex in vivo in mouse tissues hints at a potential developmental and/or physiological role for this interaction, indicating that Tesk1 may be an endogenous modulator of Spry2 function.

The constitutive binding of Tesk1 to Spry2 leads to its relocalization to Tesk1-positive vesicles including endosomes. Spry2 has previously been reported to localize to endosomes in certain studies (41), whereas others have reported a microtubular localization (25, 26). Differing amounts of endogenous Tesk1 may form the basis for this discrepancy, since in this study we have shown that Tesk1 does indeed relocalize it to endosomes.

This interaction abolishes the Grb2 binding of Spry2, resulting in the loss of its inhibition of ERK phosphorylation. Although the negative effect of Tesk1 could potentially arise from either steric hindrance or relocalization of Spry2, the lack of effect of the cytosol- and membrane-localized C100 suggests that sequestration of Spry2 to vesicles forms the basis for its negative effect. It is interesting to note that although the full-length Tesk1 localizes to vesicles, both the Tr528 and the C100 constructs show a cytosolic distribution. One possible explanation is that the targeting signal on Tesk1 is located around residue 528 and is abolished when this sequence is disrupted. However, such a signal is not immediately apparent from the primary sequence in this region. Another possibility is that two or more targeting motifs may be present in the full-length protein, including the last 100 residues, which synergize to determine the localization of the full-length protein. Thus, the individual motifs may not be sufficient for the vesicular targeting of the Tr528 and C100 constructs. The significance of the endosomal localization of Tesk1 itself remains to be determined.

The binding of Tesk1 also inhibits the Ser dephosphorylation of Spry2 by interfering with its interaction with PP2A. Although not affecting the scaffolding A subunit from binding to the N terminus of Spry2, Tesk1 displaces the catalytic C subunit from this complex. Proteins like PyST (polyoma small T antigen) have been reported to interfere with PP2A activity by displacing can potentially bind to c-Cbl and Tesk1 simultaneously, because c-Cbl binds to a region distal to the Tesk1-binding Cys-rich C terminus. Furthermore, it has been reported that the interaction between Spry2 and c-Cbl occurs both at the plasma membrane and in endosomes (36), so the relocalization of Spry2 is not exclusive to endosomes by Tesk1 may deplete only a proportion of the Spry2-c-Cbl complex.

The interaction of Spry2 with c-Cbl plays a role in the regulation of EGFR endocytosis by Spry2 (reviewed in Ref. 38). c-Cbl binds to and ubiquitinates EGFR upon ligand stimulation, leading to receptor internalization and proteasomal degradation. Several groups have reported the inhibitory role of Spry2 in this...
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A third regulatory component (subunit B) of the phosphatase (42), but interference of PP2A-A and -C binding has not been previously reported. The basis for this displacement by Tesk1 remains to be understood but may involve inaccessibility of the Spry2-PP2A-A complex in the endosomes or a physical masking of the C binding site on the A subunit.

The phosphorylation of Spry2 on conserved Ser residues has been reported in two recent studies to play a role in the stability of the protein and in its activation upon growth factor stimulation (32, 33). To date, Mnk1 and CK2 have been proposed to be the kinases that phosphorylate Spry2 on these residues. C100 seemingly affects this basal Ser phosphorylation of Spry2, but it is currently not known whether it inhibits phosphorylation by the kinases or promotes dephosphorylation by targeting Spry2 to the membrane in the resting state. It may serve as a useful tool in future studies for delineating the molecular processes that control the balance between the phosphorylation and dephosphorylation of Spry2.

Although Drosophila Spry works as a general inhibitor of receptor tyrosine kinase signaling (43), mammalian Spry isoforms seem to have evolved more selective roles. Spry2 in particular has been regarded as a positive and negative regulator of receptor tyrosine kinase signaling, owing to its opposing roles in EGFR versus other receptor tyrosine kinase signaling (38). Even in the context of EGFR signaling, the N and C termini of Spry2 have opposing effects, with the N terminus prolonging phosphorylation and dephosphorylation of Spry2 have opposing effects, with the N terminus prolonging phosphorylation and dephosphorylation of Spry2.

With Spry and Spred in its capacity as a scaffold and as a cofilin kinase could link the regulation of the Ras/ERK pathway to the modulation of the cytoskeleton, which is at the core of various cellular processes including growth, differentiation, and migration.

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