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Regulation of Sprouty2 Stability by Mammalian Seven-in-Absentia Homolog 2

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Abstract Mammalian Sprouty (Spry) gene expression is rapidly induced upon activation of the FGF receptor signaling pathway in multiple cell types including cells of mesenchymal and epithelial origin. Spry2 inhibits FGF-dependent ERK activation and thus Spry acts as a feedback inhibitor of FGF-mediated proliferation. In addition, Spry2 interacts with the ring-finger-containing E3 ubiquitin ligase, c-Cbl, in a manner that is dependent upon phosphorylation of Tyr55 of Spry2. This interaction results in the poly-ubiquitination and subsequent degradation of Spry2 by the proteasome. Here, we describe the identification of another E3 ubiquitin ligase, human Seven-in-Absentia homolog-2 (SIAH2), as a Spry2 interacting protein. We show by yeast two-hybrid analysis that the N-terminal domain of Spry2 and the ring finger domain of SIAH2 mediated this interaction. Co-expression of SIAH2 resulted in proteasomal degradation of Spry1, 2, and to a lesser extent Spry4. The related E3 ubiquitin-ligase, SIAH1, had little effect on Spry2 protein stability when co-expressed. Unlike c-Cbl-mediated degradation of Spry2, SIAH2-mediated degradation was independent of phosphorylation of Spry2 on Tyr55. Spry2 was also phosphorylated on Tyr227, and phosphorylation of this residue was also dispensable for SIAH2-mediated degradation of Spry2. Finally, co-expression of SIAH2 with Spry2 resulted in a rescue of FGF2-mediated ERK phosphorylation. These data suggest a novel mechanism whereby Spry2 stability is regulated in a manner that is independent of tyrosine phosphorylation, and provides an addition level of control of Spry2 protein levels. J. Cell. Biochem. 100: 151–160, 2007.

Key words: sprouty; FGF; ERK; proteasome; SIAH2

Spry was originally identified as an antagonist of FGF receptor signaling during tracheal development in *Drosophila* [Hacohen et al., 1998]. Loss-of-function mutations in *Spry* result in excessive tracheal branching, whereas overexpression of *Spry* results in decreased tracheal branching [Hacohen et al., 1998]. Subsequent studies in *Drosophila* reported genetic interactions between *Spry* and other receptor tyrosine kinase (RTK) pathways in several developmental contexts including wing [Reich et al., 1999] and eye development [Casci et al., 1999; Kramer et al., 1999], indicating that Spry may be a general inhibitor of RTK signaling in *Drosophila*. In mammals, four *Spry* genes were identified. Mammalian *Spry* genes are expressed in a spatially restricted but overlapping pattern during embryonic development [Minowada et al., 1999; de Maximy et al., 1999; Chambers and Mason, 2000; Zhang et al., 2001]. *Spry* genes are co-expressed in areas where several FGFs, their receptors, and another FGF signaling antagonist, Sef [Furthauer et al., 2002; Lin et al., 2002; Tsang et al., 2002], are also expressed. Mammalian *Spry* genes are implicated in the regulation of several developmental processes including branching morphogenesis of the lung [Tefft et al., 1999; Mailleux et al., 2001; Perl et al., 2003], angiogenesis [Impagnatiello et al., 2001;

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Lee et al., 2001], and limb chondrogenesis [Minowada et al., 1999].

Several studies suggest that Spry negatively regulates the Ras pathway, but the mechanism of this inhibition remains elusive [Cabrita and Christofori, 2003; Christofori, 2003; Guy et al., 2003]. All *Spry* genes have a highly conserved C-terminal cysteine-rich domain that is important for the membrane localization of *Spry* family members [Lim et al., 2000, 2002]. The N-terminal domain of *Spry* family members is less well conserved (25%–37%) and may impart unique functions to individual family members. The N-terminal domain of Spry2 binds to c-Cbl, a ring-finger domain containing protein, a protein involved in targeting the EGF receptor for proteosome-mediated degradation [Egan et al., 2002; Fong et al., 2003; Hall et al., 2003; Rubin et al., 2003]. Spry inhibits the Ras/MAP kinase pathway by inhibiting Ras [Casci et al., 1999; Hanafusa et al., 2002] or Raf activation [Reich et al., 1999; Yusoff et al., 2002]. The reasons for these discrepancies are unknown. It was shown that overexpression of Spry1 or Spry2 potentiates EGF-induced MAP kinase activation, whereas expression of the C-terminal cysteine-rich domain of Spry2 results in inhibition of EGF-induced MAP kinase activation [Egan et al., 2002]. Furthermore, the potentiating effect of Spry overexpression is mediated by sequestration of c-Cbl, thus inhibiting EGFR ubiquitination and degradation [Wong et al., 2001].

In an effort to identify additional proteins that interact with Spry and potentially regulate its function, we performed a yeast two-hybrid screen of a human lung library with the N-terminal domain of Spry2 as bait. From this screen, we identified additional Spry interacting proteins. Here, we describe the interaction of SIAH2, a mammalian homologue of the *Drosophila* seven in Absentia (*Sina*) gene with Spry2. *Sina* was originally identified as a RING finger-containing protein that plays an important role in *Drosophila* photoreceptor development [Carthew and Rubin, 1990; Carthew et al., 1994]. In genetic epistasis experiments, *Sina* acts downstream of the RTK *Sevenless* and the Ras/MAP kinase pathway. Recently, *Sina* was shown to promote ubiquitin/proteasome-dependent degradation of tramtrack, a negative regulator of neuronal differentiation in *Drosophila* [Li et al., 1997; Hirota et al., 1999]. There are three highly conserved *Sina* homologues, SIAH-1A, SIAH-1B, and SIAH-2 in mammals [Della et al., 1993; Hu et al., 1997a]. Mammalian SIAH proteins interact with several proteins including DCC (deleted in colorectal cancer) [Hu et al., 1997b], group 1 metabotropic glutamate receptors [Kammermeier and Ikeda, 2001], the adenomatous polyposis coli (APC) tumor suppressor protein [Liu et al., 2001], c-Myb [Tanikawa et al., 2001, 2000], synaptophysin [Wheeler et al., 2002], and numb [Susini et al., 2001]. Thus, SIAH polypeptides function to mediate the ubiquitin-dependent degradation of several important signaling proteins. In the present study, we found that SIAH2 regulated Spry2 protein stability. Thus, SIAH proteins add an additional level of control of RTK-signaling pathways.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen and cDNA Cloning

The N-terminal half of *Xenopus* Spry2 (xSpry-NT) containing amino acids 1–175 was cloned into pAS2-1 (Clontech) and used as bait. Y190 yeast cells were co-transformed with the bait vector and a human lung library subcloned into pACT2 (Clontech). Approximately 1.5×10^6 clones were screened, resulting in 105 positive colonies on triple dropout plates (-leu, -trp, -his) containing 5 mM 3-aminotriazole (3-AT). Putative positive clones were screened by β-galactosidase filter lift assay resulting in 56 positive clones. Individual plasmids were isolated, sequenced, and compared to GenBank databases. Several proteins were identified, one of which, SIAH2, represented 3 out of the 56 clones that were positive by β-galactosidase assay. Different fragments of SIAH2 were cloned into pACT2 and co-transformed with different fragments of xSpry2 in pAS2-1 bait plasmid, to confirm and identify the interacting domains of the two proteins.

Full-length as well as fragments of xSpry2 were cloned into either pCS2+ or pcDNA3.1/myc-his (Invitrogen). The original cDNA isolate from the yeast 2-hybrid screen lacked the first 66 amino acids and was called SIAH2-66. SIAH-66 was cloned into p3XFLAG-CMV (Sigma). SIAH1-HA, SIAH1dR-MYC, and SIAH2FL-HA were a gift from Eric Fearon (University of Michigan). SIAH2 ring finger mutant (SIAH2RM (H99A/C102A)) was a gift from Ze'ev Ronai (Mount Sinai School of Medicine, New York, NY).

Glutathione-S-Transferase (GST) Fusion Protein-Binding Assays

For fusion protein, the N-terminus of human Spry2 (hSry2) (a.a. 1–174), was cloned into pGEX-4T (Spry2-NT-GST) and transformed into *E. coli* BL21pLysS (Novagen). Bacterial cells were grown, induced with 0.1 mM IPTG, lysed by freezing and thawing three times, followed by sonication. GST-fusion proteins were purified by adsorption onto glutathione-Sepharose 4B beads. SIAH2 constructs were transcribed and translated in vitro using the TNT Coupled Reticulocyte Lysate System (Promega) in the presence of [^{35}S]-methionine and [^{35}S]-cysteine (Translabel, Amersham), according to the manufacturer's instructions. The in vitro translated proteins were mixed with the indicated GST-fusion proteins and bound to Glutathione-Sepharose beads. The beads were washed extensively in HNTG buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, containing complete protease inhibitor cocktail (Roche Molecular Biochemicals), and followed by separation on 10% or 12.5% SDS–polyacrylamide gels and autoradiography.

Cell Culture and Transfection

COS7 and 293T cells were purchased from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone). The cells were seeded into 6-well plates or 10 cm plates (Falcon), and transfected at 50%–70% confluence with the indicated combination of plasmids, using Genejuice (Novagen) as the transfection reagent according to the supplier's instructions. For some experiments, the cells were treated with the proteasome inhibitor MG132 (Calbiochem) dissolved in DMSO.

Immunoprecipitation and Western Blotting

The cells were lysed with HNTG buffer at the indicated times after transfection, and cleared of insoluble material by centrifugation for 10 min at 14,000 rpm at 4°C. The supernatants were either subjected to immunoblotting procedures directly, or immunoprecipitated with the indicated antibodies at 4°C for 2 h or overnight. Immune complexes were adsorbed to Protein A/G agarose (Santa Cruz) for 1 h followed by extensive washing with HNTG buffer. Immune complexes were eluted from the beads using 2× SDS sample buffer and heating to 95°C for 4 min. Immunoprecipitated complexes were subjected to SDS–polyacrylamide gel electrophoresis, followed by electrophoretic transfer to nitrocellulose (Schleicher & Schuell), and immunoblotting with the indicated antibodies [Kovalenko et al., 2003]. The following antibodies were used for immunoprecipitation and immunoblot analysis: rabbit anti-myc A14 (1:1,000), monoclonal anti-myc 9E10 (1:500–1:1,000), and monoclonal anti-myc 4A6 (1:5,000) (Santa Cruz and Upstate, respectively), monoclonal anti-Flag M2 (1:10,000) (Sigma) and anti-β-actin monoclonal antibody (1:5,000) (Sigma). Bound antibodies were visualized by HRP-conjugated secondary antibodies and chemiluminescence (ECL reagent, Amersham Biosciences).

RESULTS

Identification of SIAH2 as a Spry2 Interacting Protein

In order to identify additional proteins that may functionally interact with Spry2, we employed a yeast two-hybrid screen. Because Spry2 is member of the *Spry* family that consists of four members which bear a high degree of sequence homology to one another in the carboxyl-terminal cysteine-rich domain, we excluded this domain. Instead, in order to screen a human lung library, we employed the less well conserved N-terminal domain of xSpry2, (amino acids 1–174) as the bait construct (Fig. 1A). Fifty-six clones screened positive both on selective media and by β-galactosidase colony lift assays out of the 2×10^6 clones that were screened. Three clones contained a large C-terminal fragment of human SIAH2 (amino acids 66–324) (Fig. 1A). To verify the results of the initial isolation of SIAH2, we cloned SIAH2-66 (amino acids 66–324), SIAH2-ring finger (RF) (amino acids 66–153), and SIAH2 C-terminus (CT) (amino acids 151-324) into pAS2-1, as well as full-length xSpry2 (amino acids 1–314), xSpry2-CT (amino acids 175–314), and full-length xSpry2 and SIAH2 into pACT2 in order to identify the interacting domains between the two polypeptides. The results of these analyses are presented in Figure 1B. The original SIAH2 isolate (amino acids 66–324) interacted with the full-length xSpry2, as well as xSpry2-NT

but not xSpry-CT. Full-length xSpry2 interacted with SIAH2 and SIAH2-RF but not SIAH2-CT (Fig. 1B). These assays also revealed that full-length xSpry2 interacted with itself, and that this interaction was mediated through the C-terminal cysteine-rich domain of Spry2, confirming a previous report of Spry dimerization [Ozaki et al., 2005]. In addition, these data show that SIAH2 also dimerizes with itself through the ring-finger domain, an observation that has been previously reported [Hu and Fearon, 1999].

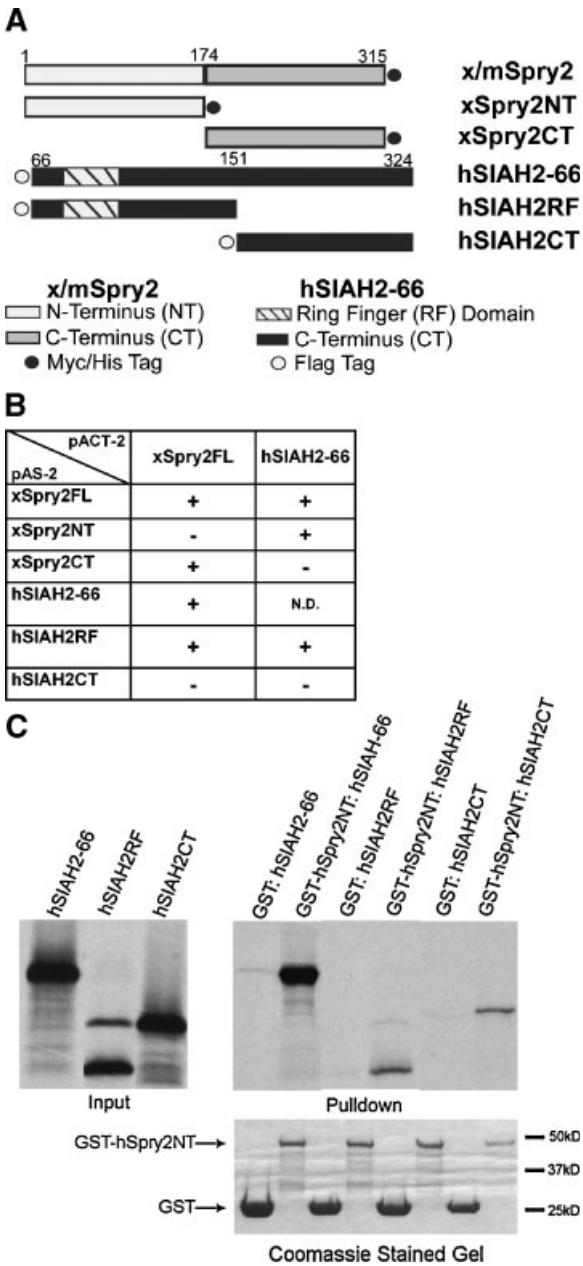

Fig. 1.

To confirm the interaction of Spry2 with SIAH2 using an independent assay system, we performed GST-pull-down experiments with in vitro translated proteins. A GST fusion protein containing human Spry2-NT (amino acids 1–174) (Spry2-NT-GST) was generated and purified. Spry2-NT-GST, or a GST control protein, was incubated with in vitro translated SIAH2-66, SIAH2-RF, or SIAH2-CT proteins. Consistent with the results of the yeast two-hybrid assays, in vitro translated SIAH2-66 interacted strongly with Spry2-NT-GST; whereas, SIAH2-RF and SIAH2-CT interacted to a much lesser extent (Fig. 1C). This suggests that SIAH2 may have a tertiary structural requirement for optimal interaction between Spry2 and SIAH2, or that SIAH2-CT potentiates the interaction of SIAH2-RF.

Interaction of Spry2 With SIAH2 Results in Proteasomal Degradation of Spry2 Protein

SIAH proteins and *Drosophila Sina* are involved in regulating protein stability by interacting with target proteins, mediating their ubiquitination and subsequent degradation by the proteasome pathway. SIAH-interacting proteins include DCC [Hu et al., 1997b], APC [Liu et al., 2001], synaptophysin [Wheeler et al., 2002], TRAF2 [Habelhah et al., 2002], and numb [Susini et al., 2001], all of which are degraded as a result of their interaction with SIAH. We thus sought to determine whether SIAH2 would mediate an increase in the turnover of Spry2 protein levels. Co-transfection of COS7 cells with SIAH2-66 expression vector and Spry2 expression vector resulted in a large reduction in Spry2 protein levels as determined

Fig. 1. Spry2 binds to SIAH2. **A**: Xenopus and mouse (x/m) Spry2 and hSIAH2 constructs used in these studies. The N-terminus of xSpry2 (xSpry2NT) was used as the ''bait'' for yeast two-hybrid assay. Subsequently, a truncated version of human SIAH2 (hSIAH2-66) was isolated, and contained residues 66-324 including the RING finger (RF) domain. Other constructs used to confirm interacting domains included xSpry2 full-length (xSpry2FL), xSpry2 C-terminal domain (xSpry2CT), and hSIAH2 C-terminal domain (hSIAH2CT). **B**: Summary of yeast-two-hybrid results. Results show that xSpry2NT interacted with SIAH2RF fragment. It was also observed that xSpry2 interacted with itself via the cysteine rich C-terminal domain (CT). **C**: In vitro transcription/translation assay. One μg of SIAH2 (−66, CT, RF) plasmid DNA was added to each coupled in vitro transcription translation reaction in the presence of [^{35}S]-methionine and [^{35}S]-cysteine. A glutathione-S-transferase (GST) fusion protein encoding human (h)Spry2 NT was used to pull-down in vitro translated hSIAH2 proteins. GST protein alone was used as a control for non-specific binding.

by immunoblot analysis (Fig. 2A). As a control, β-actin levels were unaffected by SIAH2 expression. Furthermore, SIAH2-CT and SIAH2-RF expression vectors were unable to mediate a reduction in Spry2 protein levels. These data are largely consistent with the GST pull-down experiments that indicate that there may be a tertiary structural requirement that included both SIAH2-RF and SIAH2-CT for full functional interaction with Spry2, resulting in decreased Spry2 protein levels.

Because human SIAH1 and human SIAH2 share 77% overall sequence identity [Hu et al., 1997a], and differ mainly by an amino terminal extension of 42 amino acids of SIAH2, we sought to determine the specificity of the interaction between Spry2 and SIAH2. Figure 2B shows that co-transfection of a hSIAH1 expression vector with a Spry2 expression vector had little effect on Spry2 protein levels, whereas co-transfection of a full-length hSIAH2 as well as hSIAH2-66 resulted in a substantial reduction in Spry2 protein levels. The ability of full-length SIAH2 to degrade Spry2 in a similar manner to the truncated SIAH2-66 indicated that the interaction, and subsequent degradation of Spry2 was independent of the N-terminal 65 amino acids of SIAH2. Thus, the reduction in Spry2 protein levels appears to be mediated by an interaction that involves SIAH2 but not SIAH1. To determine whether the decrease in Spry2 proteins levels when co-expressed with SIAH2 is via the proteasome, we employed the proteasome inhibitor MG132. The addition of MG132 blocked the decrease in Spry2 protein levels when SIAH2 was co-expressed, suggesting that the decrease is proteasome-dependent (Fig. 2C).

To determine whether the effect of SIAH2 on Spry2 levels extends to other members of the Spry family, a SIAH2 expression vector was co-transfected with expression vectors for murine Spry1, Spry2, and Spry4 into COS7 cells. Co-transfection of SIAH2 with murine Spry1, 2, and 4 resulted in a decrease in protein levels (Fig. 3A). The level of Spry4 appears to be less affected by SIAH2; however, the protein stability without SIAH2 appears to be higher than Spry1 or Spry2 (Fig. 3A). These data suggest that despite several reports indicating unique roles of each mammalian Spry member, all are targeted to the proteasome for degradation, and they may contain similar structural requirements for their interaction with SIAH2.

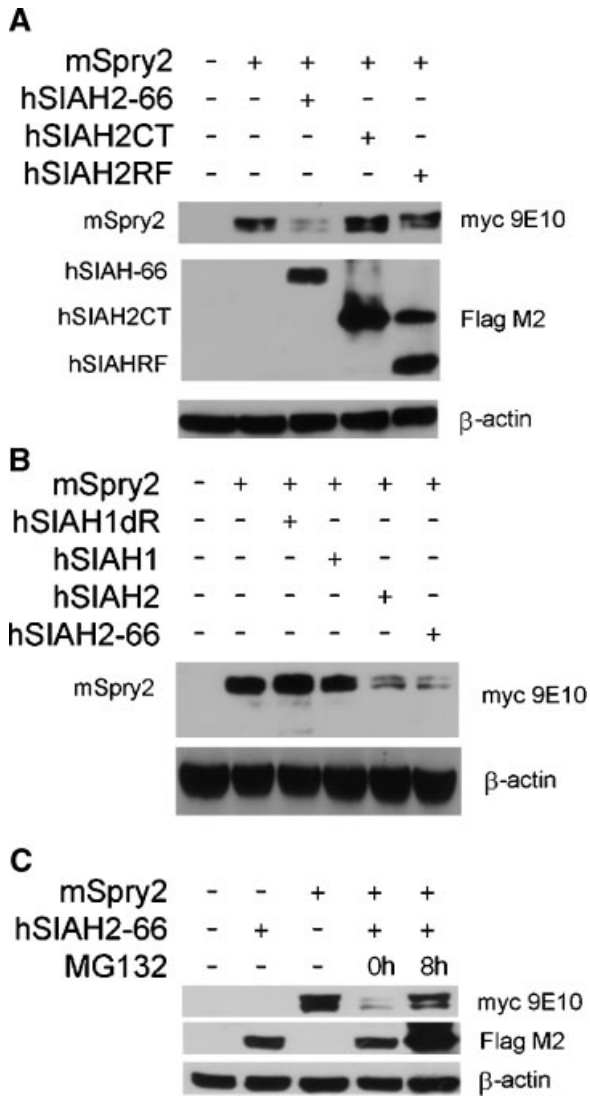

Fig. 2. SIAH2 targets Spry2 for proteasomal degradation. **A**: SIAH2 reduces Spry2 stability in transfected cells. Epitope-tagged mSpry2 was transfected alone or with Flag-tagged SIAH2-66 into COS7 cells. Lysates were prepared and subjected to immunoblot with anti-myc 9E10 monoclonal antibodies to detect transfected mSpry and Flag M2 monoclonal antibodies to detect transfected SIAH2 or deletion mutants of SIAH2. Immunoblots were reprobed with monoclonal β-actin antibodies to demonstrate that equal amounts of lysate were loaded in each lane. **B**: SIAH2 but not SIAH1 reduces Spry2 stability in transfected cells. COS7 cells were transiently transfected with myc/his-tagged Spry2 with or without SIAH1 full-length (FL), or the deleted RING finger domain (dR), and SIAH2-66 or SIAH2FL. Cell lysates were prepared and subjected to immunoblot analysis with anti-myc (9E10) monoclonal antibodies to detect transfected Spry2. Immunoblots were reprobed with anti-β-actin antibodies to demonstrate equivalence of loading. **C**: Spry2 is degraded via the proteasome pathway. COS7 cells were transiently transfected with myc/his-tagged Spry2 and flag-tagged SIAH2 constructs. Eighteen hours post-transfection, 50 μM MG132 (a 26S proteasome inhibitor) was added. Cell lysates were collected at 0 and 8 h after the addition of the drug, with DMSO added to as a vehicle control. Lysates were then subjected to immunoblot analysis with the antibodies indicated on the right.

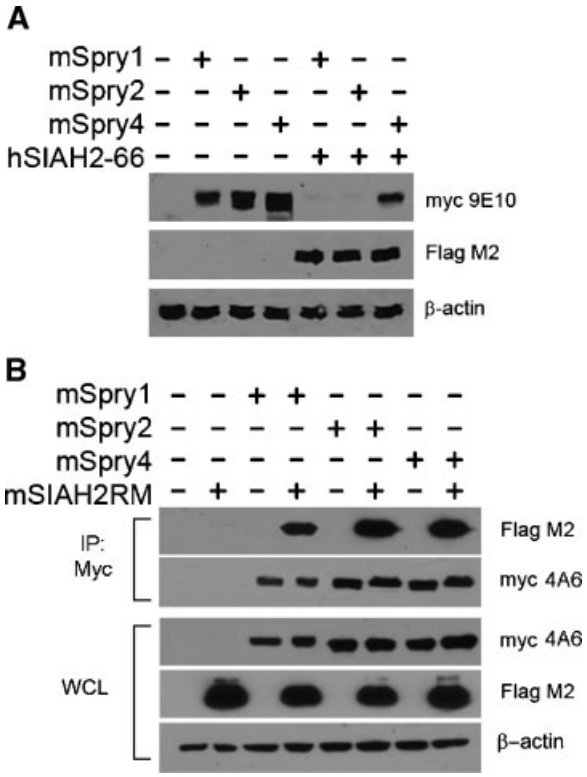

Fig. 3. SIAH2 binds to and targets Spry1, 2, and 4 for degradation. **A**: Spry1, Spry2, and Spry4 are degraded in the presence of SIAH2. COS7 cells were transiently transfected with myc/his-tagged Spry 1, Spry2, or Spry4 with or without flag-tagged SIAH2-66 or SIAH2RM, and subjected to immunoblot analysis with the antibodies indicated on the right. Results show that Spry1, Spry2, and to a lesser extent Spry4 were degraded by SIAH2. **B**: Spry1, 2, and Spry4 co-immunoprecipitate with an SIAH2 RING finger mutant (mSIAH2RM). COS7 cells were transfected with myc/his-tagged Spry1, Spry2, and Spry4 in the presence or absence of flag-tagged SIAH2RM. After 24 h, cell lysates were prepared subjected to immunoprecipitation with a rabbit ant-myc antibody (Santa Cruz). Immunoprecipitated proteins were separated by SDS–PAGE followed by immunoblotting with anti-myc monoclonal antibodies (myc 4A6) to detect Spry proteins, and Flag M2 monoclonal antibodies to detect SIAH2RM. To demonstrate levels of protein expression from transfected plasmids, whole cell lysates (WCL) were immunoblotted with anti-myc 4A6, Flag M2, and β-actin antibodies as a loading control.

Amino acid sequence comparisons between Spry family members indicate that Spry1 and Spry2 are more similar to one another than either one is to Spry4. Since mammalian Spry protein levels are decreased by co-expression with SIAH2, it is likely that Spry family members are able to bind SIAH2. In order to determine whether SIAH2 and Spry1, 2, and 4 interact in vitro, we acquired an SIAH2 mutant (SIAH2RM) in which the ring finger activity has been ablated by point mutations (H99A\C102A) [Habelhah et al., 2002]. This mutation abolishes the E3 ligase activity of SIAH2 and increases its stability versus wild-type SIAH2 [Habelhah et al., 2002]. SIAH2, like SIAH1, is able to catalyze self-ubiquitination and regulates its own stability [Hu and Fearon, 1999]. Because SIAH2 is able to degrade Spry, as well as itself, determining their interaction would be difficult to establish. In order to further determine the interaction between SIAH2 and Spry1, 2, and 4, co-immunoprecipitation was performed using SIAH2RM, which demonstrates a higher stability in cells and is unable to degrade Spry. SIAH2RM was able to co-immunoprecipitate with Spry1, 2, and 4 with relatively equal ability (Fig. 3B).

SIAH2-Mediated Degradation of Spry2 is Phosphotyrosine-Independent

Spry2 interacts with c-Cbl, a known regulator of RTK signaling [Wong et al., 2001]. The interaction of Spry2 and c-Cbl is dependent upon tyrosine phosphorylation of Spry2 at Tyr55, which acts as a docking site for the SH2 domain of c-Cbl [Fong et al., 2003; Hall et al., 2003; Rubin et al., 2003]. Ordinarily, c-Cbl binds to phosphorylated tyrosine residues on EGFR which results in the ubiquitination, endocytosis, and proteasome-dependent degradation of the EGFR. The interaction of tyrosine phosphorylated Spry2 with c-Cbl has several consequences, one of which is that Spry2 sequesters c-Cbl away from the EGFR. This prevents ubiquitination and degradation of the EGFR, resulting in sustained signaling from the EGFR. Another consequence of the Spry2-Cbl interaction is the poly-ubiquitination and subsequent degradation of Spry2 via the proteasome. To determine whether SIAH2-mediated degradation of Spry1 and Spry2 is dependent on tyrosine phosphorylation, we prepared dominant negative mutants of Spry1 (Y53F) and Spry2 (Y55F), as described [Sasaki et al., 2001]. Spry1Y53F and Spry2Y55F protein levels decreased when co-expressed with SIAH2 in a manner similar to wild-type Spry1 and Spry2 (Fig. 4A). Furthermore, mutation of an additional tyrosine on the carboxy terminus, Y227F, which was shown to also affect the ability of Spry2 to attenuate FGF-induced MAPK activation [Rubin et al., 2005], was also degraded by SIAH2. The data presented here suggest that Spry1 and Spry2 protein levels may be regu-

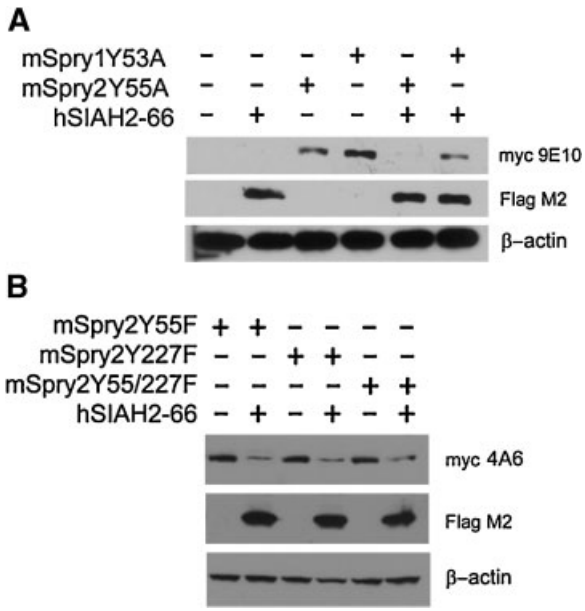

Fig. 4. Degradation of Spry1 and Spry2 by SIAH2 is independent of the conserved N-terminal tyrosine residues of Spry1 and Spry2. **A**: The conserved N-terminal tyrosine of Spry1 and Spry2 is dispensable for SIAH2-mediated degradation of Spry1 and Spry2. COS7 cells were transfected with expression vectors encoding Spry1Y53A or Spry2 Y55A in the presence or absence of SIAH2 for 24 h. Cell lysates were prepared and analyzed by immunoblot analysis with the antibodies indicated on the right. Results indicate that the degradation of Spry1 and Spry2 are independent of the conserved N-terminal Tyr residue. **B**: Other Tyr residues of Spry2 that are targets of tyrosine phosphorylation are dispensable for SIAH2-mediated degradation of Spry2. Expression plasmids encoding Spry2Y55F, Spry2Y227F, or Spry2Y55/227F were transfected with or without SIAH2 for 24 h. Cell lysates were then prepared and subjected to immunoblotting with the antibodies indicated on the right. The results demonstrate that degradation of Spry2 Tyr mutants occurred in a manner similar to wild-type Spry2.

lated by an SIAH2-dependent mechanism that is independent of tyrosine phosphorylation of Spry1 or Spry2. The protein stability of Spry2 tyrosine mutants was decreased in a similar manner to wild-type Spry2 (Fig. 4B). Spry4 was not examined, since the function of its conserved Tyr at residue 53 remains to be determined. These results demonstrate a novel mechanism in which SIAH2 was able to regulate the protein stability of Spry2, independent of tyrosine phosphorylation.

SIAH2 Decreases the Inhibitory Effect of Spry2 on FGF-Induced ERK Phosphorylation

Since Spry2 inhibits ERK phosphorylation by multiple mechanisms that occur either upstream of Ras [Casci et al., 1999; Hanafusa et al., 2002], or at the level of Raf-1 [Reich et al., 1999; Yusoff et al., 2002], we sought to determine whether SIAH2 overexpression with Spry2 would relieve the inhibitory effects of Spry2 on FGFR-mediated ERK1/2 phosphorylation. Transfection of 293T cells with Spry2 resulted in an FGF2-induced decrease in ERK phosphorylation (Fig. 5). Overexpression of SIAH2 alone had no effect on FGF-mediated ERK phosphorylation and ERK protein levels (Fig. 5). Co-transfection of SIAH2 and Spry2 resulted in a rescue of FGF2-induced ERK phosphorylation, and correlated with decreased Spry2 protein levels (Fig. 5).

DISCUSSION

The reduction in Spry2 protein via interaction with c-Cbl serves to limit the duration of the inhibitory action of Spry2 on FGF- and EGF-induced MAPK activation [Fong et al., 2003; Hall et al., 2003; Rubin et al., 2003]. On the basis of these data, it was hypothesized that the presence or relative amount of c-Cbl may act as a switch between inhibitory or stimulatory functions of Spry2. The cytoplasmic tyrosine

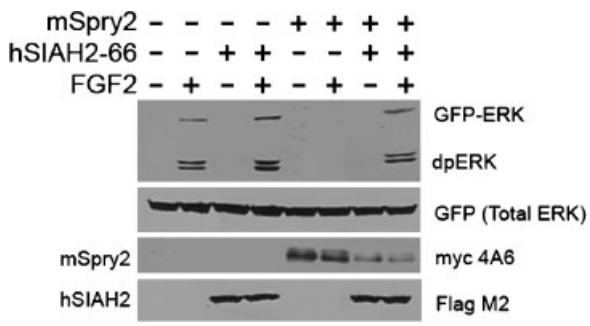

Fig. 5. SIAH2-mediated degradation of Spry2 relieves Spry2-mediated inhibition of FGF-induced ERK phosphorylation. In order to determine whether forced expression of SIAH2 attenuates the inhibitory action of Spry2 on FGF-induced ERK phosphorylation, 293T cells were transiently transfected with myc/his-tagged Spry2, flag-tagged SIAH2, as indicated. For each condition, cells were transfected with a GFP-ERK fusion protein. Eighteen hours post-transfection the cells were serum starved for 6 h followed by addition of 25 ng/ml FGF2 for 30 min. Cell lysates were prepared and subjected to immunoblot analysis. GFP-ERK shows the phosphorylation of transfected GFP-ERK in response to FGF stimulation, and dpERK (Sigma) shows the phosphorylation of endogenous ERK. Blotting for GFP shows equivalence of transfection of GFP-ERK as well as serving as a loading control. The levels of Spry2 protein were shown by blotting with anti-myc antibodies (4A6), and the expression of SIAH2 was shown by blotting with Flag M2 antibodies. The results show that SIAH2 relieved the inhibition of FGF-mediated phosphorylation of both endogenous ERK, as well as transfected GFP-ERK.

kinase Src was implicated in Spry2 tyrosine phosphorylation [Li et al., 2004]; however, other tyrosine kinases may also be involved [Rubin et al., 2005]. SIAH2 may serve as an additional mechanism to regulate Spry2 levels and decrease the inhibitory effects of Spry2 on MAPK signaling. The E3 ubiquitin ligase function of SIAH2 is well characterized, and appears to occur in cells that are under conditions of stress. SIAH2 decreases tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2), which acts to regulate the cellular response to stress and cytokines through the regulation of stress signaling cascades [Habelhah et al., 2002]. Additionally, SIAH2 was shown to play a role in hypoxia, as it regulates the turnover of prolyl hydroxylases (PHDs), which hydroxylate hypoxia inducible factor 1 alpha (HIF1alpha) during normoxia to prevent transcriptional activation [Nakayama et al., 2004]. The role of Spry2 in cellular stress-activated conditions has not been elucidated. The degradation of Spry2 by SIAH2 may serve to increase basal MAPK activity in the absence of RTK ligand activation, and the subsequent absence of Spry2 tyrosine phosphorylation, to allow for cells to repair and proliferate following stress or injury. The regulation of Spry expression and activity under cellular stress conditions will be the subject of future investigations.

Although we clearly demonstrated that co-expression of SIAH2 with Spry2 resulted in decreased Spry2 protein levels, and this was inhibited by the proteasomal inhibitor MG132, we were unable to detect poly-ubiquitination of Spry2 (data not shown). These data suggest that while Spry2 can interact with SIAH2 through protein–protein interactions, it is possible that a third protein may be part of this complex which becomes ubiquitinated and targets the complex to the proteasome. Spry family members interact with key MAPK-signaling pathway molecules including Grb2, SOS, Raf1, and Shp2 [Hanafusa et al., 2002; Sasaki et al., 2003; Ozaki et al., 2005]. The relative levels of these proteins were unchanged when Spry2 and SIAH2 were overexpressed in COS7, 293T, or HT1080 cells (data not shown). This suggests that a previously unidentified Spry2 interacting protein may be part of the complex, which may become poly-ubiquitinated, which then targets the complex for degradation by the proteasome. Alternatively, ubiquitinated SIAH2 in complex with Spry2 may be sufficient to target Spry2 for proteasomal degradation. The ability of SIAH2 to mediate degradation Spry2 relieved inhibition of FGF signaling, and provided a functional regulation of Spry2 protein levels in a phosphotyrosine-independent manner (Fig. 6).

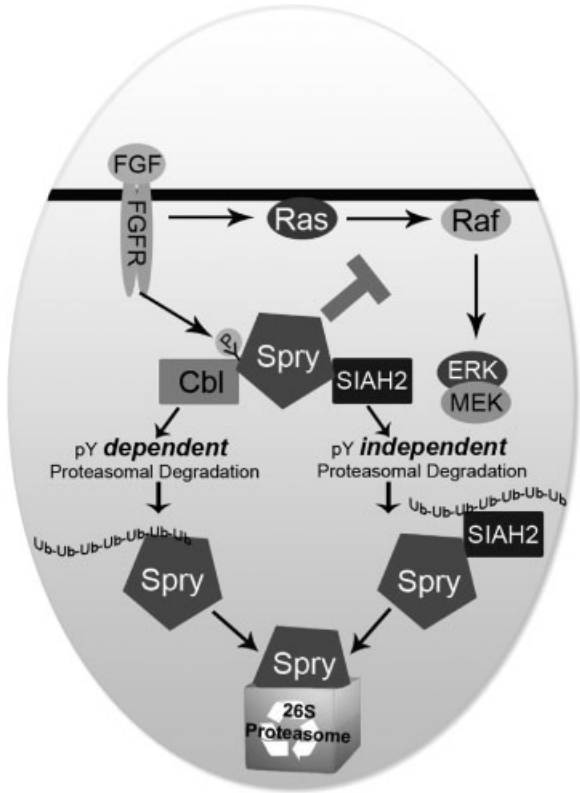

Fig. 6. Scheme for two cellular mechanisms by which Spry2 levels are regulated. This diagram is a representation of Spry2 inhibition of FGF-induced ERK phosphorylation, and the regulation of Spry2 levels in cells. In previously published reports, Spry2 becomes tyrosine phosphorylated on a conserved N-terminal tyrosine residue resulting in an interaction between Cbl and Spry2 culminating in proteasomal degradation of Spry2. Results in the present study indicate that SIAH2 physically associated with Spry2 and resulted in proteasomal degradation of Spry2. These results indicate that the interaction of Spry2 with SIAH2 adds an additional level of regulation to FGF-mediated ERK activation as well as cellular levels of Spry2.

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