Mammalian Sprouty Proteins Inhibit Cell Growth and Differentiation by Preventing Ras Activation*

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Isabelle Gross, Bhramdeo Bassit, Miriam Benezra, and Jonathan D. Licht‡
From the Derald H. Ruttenberg Cancer Center and Department of Medicine, Box 1130, Mount Sinai School of Medicine, New York, New York 10029

Sprouty was genetically identified as an antagonist of fibroblast growth factor signaling during tracheal branching in Drosophila. In this study, we provide a functional characterization of mammalian Sprouty1 and Sprouty2. Sprouty1 and Sprouty2 inhibited events downstream of multiple receptor tyrosine kinases and regulated both cell proliferation and differentiation. Using NIH3T3 cell lines conditionally expressing Sprouty1 or Sprouty2, we found that these proteins specifically inhibit the Ras/Raf/MAP kinase pathway by preventing Ras activation. In contrast, activation of the phosphatidylinositol 3-kinase pathway was not affected by Sprouty1 or Sprouty2. We further showed that Sprouty1 and Sprouty2 do not prevent the formation of an SNT-Grb2-Sos complex upon fibroblast growth factor stimulation, yet block Ras activation. Taken together, these results establish mammalian Sprouty proteins as important negative regulators of growth factor signaling and suggest that Sprouty proteins act downstream of the Grb2-Sos complex to selectively uncouple growth factor signals from Ras activation and the MAP Kinase pathway.

Normal development requires precise spatial and temporal regulation of signal transduction pathways involved in cell growth and differentiation. Negative control of growth factor response is achieved both by restriction of the incoming signal itself and induction of counter regulatory mechanisms affecting the propagation of the signal. The expression of many inhibitors are induced by the pathway they eventually antagonize, providing the potential for a tight autoregulation (for a review, see Ref. 1). Recently, sprouty (spry) was identified by genetic studies as such an inhibitor (2).

Spry was originally described as an antagonist of Breathless FGF receptor signaling during tracheal branching in Drosophila. Loss of function mutations of spry led to excessive FGF signaling and ectopic branching, whereas engineered overexpression of spry blocked the branching (2). As other groups reported genetic interactions between spry and several different receptor tyrosine kinases (RTK) in multiple contexts, it became clear that spry was a general inhibitor of RTK signaling during Drosophila development (3–6). Through a data base search, three human genes were identified with sequence similarity to Drosophila spry (2) and a fourth family member was described in the mouse (7). Mammalian spry genes are expressed in highly restricted patterns in the embryo during early development and in many adult tissues (7–9). In most tissues, the different family members appear to be co-regulated and their expression shows a close correlation with known sites of FGF signaling. Mammalian Spry proteins may be key regulators of several developmental processes, including lung branching morphogenesis, midbrain and anterior hindbrain patterning, and limb chondrocyte differentiation (8–10).

Genetic and biochemical analysis performed by Casci et al. (3) suggested that Drosophila Spry negatively regulates the Ras pathway, but the molecular mechanism of this inhibitory activity was not determined (5). All Spry proteins share a unique, highly conserved, cysteine-rich C-terminal domain. This domain was shown to be necessary for the membrane translocation of Spry by a yet unknown mechanism (2, 3, 11). The N-terminal portion of the Spry proteins is less conserved as it exhibits only 25–37% identity among the different mouse family members. These sequence differences could be responsible for functional divergence among the Spry proteins. In particular, the size difference between Drosophila and much smaller mammalian Spry N-terminal regions is intriguing.

Our laboratory has studied the Wilms Tumor 1 (WT1) gene, a tumor suppressor gene involved in embryonic kidney development, for several years. We performed a representative difference analysis screen to isolate transcriptional target genes of WT1. One of the genes identified was mouse spry1. To model the role of mammalian Spry during development and tumorigenesis, we established stable inducible Spry1 and Spry2 NIH3T3 cell lines. We demonstrated that Spry1 and Spry2 antagonized growth factor signaling by specifically inhibiting the Ras/Raf/MAP kinase pathway. We methodically examined the inhibitory effect of Spry on the different components of the signal transduction cascade and identified the activation of Ras as the target of Spry activity. We showed that Spry1 and Spry2 can inhibit both proliferation of NIH3T3 cells and differentiation of PC12 cells. These results suggest that Spry proteins, by limiting RTK signaling, play an important role in development and growth control.

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†To whom correspondence should be addressed. Tel.: 212-659-5487; Fax: 212-849-2523; E-mail: jonathan.licht@mssm.edu.
‡To whom correspondence should be addressed. Tel.: 212-659-5487; Fax: 212-849-2523; E-mail: jonathan.licht@mssm.edu.

The abbreviations used are: FGF, fibroblast growth factor; RTK, receptor tyrosine kinases; nt, nucleotide(s); PDGF, platelet-derived growth factor; CS, calf serum; NGF, nerve growth factor; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; PI 3-kinase, phosphatidylinositol 3-kinase; MAP kinase, mitogen-activated protein kinase; RBD, Ras-binding domain; GAP, GTPase activating protein.
EXPERIMENTAL PROCEDURES

Plasmids—The nucleotide (nt) positions for murine spry1 and spry2 are as listed under AF176903 and AF176905, respectively. The mouse spry1 cDNA was reconstituted by ligation of a StuI polymerase chain reaction fragment (nt 402–1007)2 to an EST fragment (GenBankTM AA951484, nt 982–2489) in the pSPORT1 vector (Life Technologies). Nt 481–1469 of spry1 were subcloned EcoRI-XhoI in-frame with the Flag tag in the pc2FH vector (gift of D. Sassoil). Mouse spry2 cDNA was isolated by reverse transcriptase-polymerase chain reaction using primers to human spry2 (nt 391–411, nt 1330–1350 of GenBankTM AF039843) and RNA extracted from mouse podocytes (15). The EcoRI-XhoI fragment was subcloned in the pc2FH vector. Flag-Spry1 or Flag-Spry2 were used as polymerase chain reaction templates to subclone spry1 and spry2 into the pCEV29 vector (14). For the Tet-off Spry expression system, Flag-Spry1/2 fragments were subcloned into the ptRE vector (CLONTECH). Flag-tagged spry1 (nt 1–1469) or spry2 (nt 1–1245) were subcloned into the MigRI vector (15) to obtain Spy1/2-ires-GFP. The Myc-Grb2 construct was made by linking nt 79–729 of human geb2 (GenBankTM NM-002086) to the Myc tag within pcDNA3-1/Myc-His (Invitrogen). SRE-Luc (R. Prywes) contains nt −355 to −297 of the murine c-fos promoter (16). NF-kB-Luc (A. Chan) contains 3 NF-kB elements in the pGL2 Luciferase (CLONTECH) (17). All novel constructs were sequenced.

Cell Culture, Growth Factors, and Transfection Methods—NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and transfected with LipofectAMINE Plus (Life Technologies). PC12 cells were grown on tissue culture dishes coated with poly-l-lysine (0.001%, Sigma) in Dulbecco’s modified Eagle’s medium containing 10% calf serum and 0.5 mg/ml tetracycline. Spry1 and Spry2 Tet-off NIH3T3 cell lines were established by transfection of the Tet-off cells with pTRE-Spry1 or pTRE-Spry2 or an empty vector and selection in 0.5 μg/ml G418-sulfate (Roche Molecular Biochemicals). 293T cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum transfected with Superfect (Qiagen Inc.). All NIH3T3 cells exhibiting neurite outgrowth (twice the diameter of the cell) among the GFP positive cells.

Immuno blot Analysis—Cells were lysed (20 μM HEPES, pH 7.5, 10 μM EDTA, pH 8, 40 μM β-glycerophosphate, 1% Nonidet P-40, 2.5 mM MgCl2, 2 mM sodium orthovanadate, and one tablet of Complete™ protease inhibitors (Roche Molecular Biochemicals) for 50 μl). 20–50 μg of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The following primary antibodies were used: FLAG M2 mouse monoclonal (Sigma); phospho-p44/42 MAP kinase (Thr202/Tyr204) E10 mouse monoclonal (NEB); Erk2 (K-23) rabbit polyclonal (Santa Cruz); phospho-Akt (Ser473) rabbit polyclonal (New England Biolabs); Akt rabbit polyclonal (New England Biolabs); phospho-Gsk-3β (Ser9) rabbit polyclonal (New England Biolabs); Myc-Grb2 mouse monoclonal (Transduction Laboratories); Ras clone Ras10 mouse monoclonal (Upstate Biotechnology); Tyr(P) clone 4G10 mouse monoclonal (Upstate Biotechnology); FRS2 (H-91) rabbit polyclonal (Santa Cruz); c-Myc clone 9E10 mouse monoclonal (Santa Cruz); GST (Z-5) rabbit polyclonal (Transduction Laboratories). Al5 mouse monoclonal (Bethesda Research Laboratories); C-Raf1 rabbit polyclonal (Santa Cruz); Grb2 mouse monoclonal (Transduction Laboratories). For the detection, we used one of the following conjugated antisera: peroxidase goat anti-rabbit IgG (H + L) (Roche Molecular Biochemicals) at 1/7000; peroxidase goat anti-mouse IgG (H + L) (Roche Molecular Biochemicals) at 1/7000; Finally, the membranes were developed using ECL (Amersham Pharmacia Biotech).

RESULTS

Endogenous Spry Expression Is Regulated by Growth Factors in NIH3T3 Cells—To characterize mammalian Spry, we chose NIH3T3 cells which have been extensively utilized as a model for cell proliferation, oncogenesis, and growth factor signaling. We first examined the expression of spry1 and spry2 in proliferating NIH3T3 cells (Fig. 1A). Northern blot analysis revealed a major transcript of about 2.5 kilobases for both genes but occurred without protein synthesis (data not shown).

Endogenous spry1 was downregulated (about 40%) and data not shown). These results show that endogenous spry1 and spry2 are differentially regulated by growth factors in NIH3T3 cells, suggesting that they may play specific roles in these cells. In addition, as
spry2 expression was inhibited by Spry proteins (Fig. 4D). In contrast, c-fos expression was rapidly induced upon serum or FGF treatment (Fig. 4D). However, in the presence of Spry1 or Spry2, c-fos expression was significantly blocked. Compared with the control NIH3T3 Tet-off cell line, c-fos expression stimulated by serum was reduced by about 30%. More strikingly, c-fos expression induced by FGF was decreased by 70% (Spry1) to 80% (Spry2) (Fig. 4D). Thus, this set of experiments shows that mammalian Spry1 and Spry2 are able to inhibit the transcriptional events mediated by growth factor signaling and the induction of a gene required for DNA synthesis and cell division.

Spry1 and Spry2 Specifically Inhibit the Erk1/2 MAP Kinase Pathway—We used the Spry inducible cell lines to examine the effect of Spry1 and Spry2 on the Ras/Raf/MAP kinase and the phosphatidylinositol 3-kinase (PI 3-kinase) pathways, which are two major pathways mediating growth factor signaling in NIH3T3 cells (for review, see Ref. 27). In the presence of Spry1 or Spry2, FGF or PDGF-mediated activation of the Erk1/2 MAP kinases, as visualized by phospho-specific antibodies, was strikingly inhibited (Fig. 5A and B). No such effect was found upon tetracycline withdrawal in the control cell line (Fig. 5A and B). A time course of FGF stimulation showed that in the presence of Spry1 or Spry2, the stimulation of Erk1/2 was also delayed, with the peak of phosphorylated MAP kinases accumulation occurring at 10 min rather than 3 min after stimula-
In contrast, PDGF-mediated activation of the key protein of the PI 3-kinase pathway, the serine/threonine kinase Akt, was not inhibited in the presence of Spry proteins (Fig. 5D). Moreover, activation of a downstream target of Akt, the serine/threonine Gsk-3β, was also not affected (data not shown). A time course of PDGF stimulation indicated that Spry proteins did not inhibit Akt activation at any specific time point (Fig. 5E). These results show that mammalian Spry1 and Spry2 do not inhibit the PI 3-kinase pathway but specifically inhibit the mitogen-activated protein kinase (MAP kinase) activation was less sustained.

**FIG. 2.** Spry1 and Spry2 can inhibit proliferation of NIH3T3 cells and block DNA synthesis. A and B, colony suppression assay. NIH3T3 cells were transfected by an empty expression vector (pCEV29), pCEV29-Spry1 or pCEV29-Spry2 for 3 days before dilution and selection for 2 weeks in G418. Colonies were stained with Giemsa and counted. Representative plates are shown (A) and the data are summarized in the table (B). The number of colonies obtained in three independent experiments was averaged and compared. The results are also presented as average ratio between the number of colonies obtained with pCEV29-Spry1 or pCEV29-Spry2 and the number of colonies obtained with pCEV29, this being set at 100%. The S.D. represents the standard deviation of the ratio observed between three independent experiments. C, apoptosis was detected by annexin V-fluorescein isothiocyanate labeling in NIH3T3 cells transfected by bax but not by spry1 or spry2. Transfected cells were identified by co-transfection of a vector encoding a red fluorescent protein. D, expression of Spry1 and Spry2 upon tetracycline removal in the NIH3T3 Tet-off inducible cells was analyzed by immunoblot. The cells were serum starved (0.2% CS for 24 h with tetracycline) before stimulation with 10% CS in the presence (+ Tet) or absence (− Tet) of tetracycline for 24 h. The anti-Flag tag antibody was used to visualize Spry1 and Spry2. E, [3H]thymidine incorporation assay. Triplicate plates of control (Ct), Spry1 or Spry2 NIH3T3 inducible cells were treated as described in D and [3H]thymidine was added during the last 4 h of serum stimulation. A representative experiment is shown but equivalent results were obtained in independent experiments and with different Spry clones (data not shown). To facilitate comparison, the average serum stimulation (the ratio between the amount of [3H]thymidine incorporated with 10% versus 0.2% serum) in the presence of tetracycline was set at 100% for each clone. The average serum stimulation in the absence of tetracycline was calculated and compared with the one obtained in the presence of tetracycline. Error bars correspond to the standard deviation (in %) observed among the triplicates.

**FIG. 3.** Spry1 and Spry2 can inhibit differentiation of PC12 cells. Proliferating PC12 cells were transfected with an IRES-GFP control plasmid (Ct), a Spry1-IRES-GFP plasmid (Spry1), or a Spry2-IRES-GFP plasmid (Spry2). After 48 h, differentiation was induced with NGF (50 ng/ml) or bFGF (20 ng/ml). Neurite outgrowth was examined after 3 days and quantified. A, representative fields observed with phase-contrast microscopy showing equivalent differentiation in the different plates (magnification × 100). B, representative transfected cells observed with fluorescence microscopy are shown (magnification ×200). C, differentiation of transfected cells was quantified by examination of the GFP positive cells. The values correspond to the average % of differentiated cells ± S.D. obtained in three independent experiments.
Erk1/2 MAP kinase pathway. They also imply that in the presence of Spry proteins, some signals downstream of the RTK can be propagated.

**Spry1 and Spry2 Prevent Ras Activation**—We next determined the effect of Spry proteins on the stepwise activation of the different components of the MAP kinase pathway upon growth factor treatment. We examined the activation of the dual-specificity MAP kinase kinases Mek1/2 which phosphorylate Erk1/2, stimulating their activity in response to growth factor treatment. As seen in Fig. 6A, Mek1/2 phosphorylation...
was dramatically reduced in the presence of Spry1 or Spry2, indicating that the signal was blocked upstream of Mek1/2, possibly at the level of the MAP kinase kinase kinase Raf-1. Therefore, we directly examined Raf-1 kinase activity. Fig. 6B shows that in the presence of Spry2, the ability of immunoprecipitated Raf-1 from FGF-stimulated cells to phosphorylate a recombinant GST-Mek1 protein was greatly reduced. This indicates that Raf-1 is inactive in the presence of Spry proteins and suggests that Spry act upstream of Raf-1.

The steps leading to Raf-1 activation are not fully understood but require binding to Ras (for a review, see Ref. 27). Therefore, we examined the binding of Raf-1 to Ras in the Spry cell lines (28). Fig. 6C shows that the amount of endogenous Ha-Ras bound by recombinant GST-Raf-1 Ras-binding domain (RBD) fusion protein upon FGF treatment was greatly reduced in the presence of Spry1 or Spry2. This could be due to either a direct inhibition of the Ras/Raf-1 interaction or to an inhibition of Ras activation, since only activated, GTP-bound Ras, can bind Raf-1. To discriminate between these two possibilities, we examined the binding of a constitutively active form of Ras (Ha-Ras R12) to Raf-1 in the presence of Spry1 or Spry2. We performed the same GST-Raf-1/Ras binding assay with lysates of NIH3T3 cells transfected with Ras and Spry1 or Spry2 expression vectors. As presented in Fig. 6D, expression of Spry1 or Spry2 reduced the binding of wild type Ha-Ras (induced by FGF) to GST-Raf-1, but had no significant effect on the binding of the constitutively active Ha-Ras R12. This suggests that Spry proteins do not interfere directly with the binding of Ras to Raf-1, but rather inhibit the activation of Ras.

Finally, we examined the phosphorylation of the adaptor FR52/SNT-1, which is the primary substrate of the activated FGF receptor (29, 30) and thus reflects the activity of the RTK. Fig. 6E graphically shows that the ability of the FGF receptor to mediate phosphorylation of FR52/SNT-1 after FGF stimulation was not inhibited in the presence of Spry proteins while at the same time, the activation of the Erk1/2 MAP kinases was almost completely blocked. This indicates that the ability of the FGF receptor to phosphorylate a downstream substrate and transduce the growth factor signal is intact and that Spry proteins act downstream of the receptor to inhibit signaling.

Spry Proteins Do Not Prevent the Recruitment of Sos to the RTK Signaling Complex—Since Spry expression did not inhibit the ability of a RTK to generate a signal, we hypothesized that the ability of Spry to inhibit Ras activation would involve its binding to one, or several, more downstream component(s) of the Ras pathway at the inner surface of the plasma membrane. We attempted to co-immunoprecipitate Spry1 or Spry2 with several putative partners from lysates of co-transfected

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**Fig. 6. Spry1 and Spry2 act upstream of Ras.** A–C, control (Ct), Spry1 or Spry2 NIH3T3 inducible cells were serum starved (0.3% CS for 24 h) in the presence (+Tet) or absence (−Tet) of tetracycline before stimulation with bFGF (20 ng/ml in A and 50 ng/ml in B, C, and E). After stimulation (15 min in A and 2 min in B, C, and E), the cells were lysed and proteins extracted. Phosphorylation of Erk1/2 was always checked and found to be decreased upon Spry expression. Each experiment was repeated at least twice with similar results being obtained and intensities of the signal were measured using NIH Image software. A, proteins were analyzed by successive immunoblotting with an antibody directed against phosphorylated Erk1/2 (P-Erk1/2), and an antibody directed against phosphotyrosine (P-Tyr). MAP kinase activation in the cell extracts was also examined by immunoblotting using an antibody directed against the Myc tag. E, proteins were incubated with an FRS2/SNT-1 antibody and the immunoprecipitates were analyzed by immunoblotting using an antibody directed against phosphotyrosine (P-Tyr). MAP kinase activation in the cell extracts was also examined by immunoblotting with an antibody directed against phosphorylated Erk1/2 (P-Erk1/2).
NIH3T3 cells or from the Spry inducible cell lines. We were not able to find any physical interaction between Spry1 or Spry2 and endogenous Ha-Ras, Raf-1, and Ras GAP (31) (data not shown). We also failed to find any interaction between recombinant GST-Ras or GST-Raf-1 proteins and Spry proteins from the inducible cell lines (data not shown). Finally, we tested Grb2, the adaptor protein which allows the recruitment of the GDP/GTP exchange factor Sos upon stimulation (32). As shown of Fig. 7A, the Grb2 and Spry2 proteins were readily co-immunoprecipitated from lysates of co-transfected NIH3T3 cells. Surprisingly, in a simultaneous experiment under the same conditions, we could not co-immunoprecipitate the Grb2 and Spry1 proteins (Fig. 7B).

Since Spry2 co-immunoprecipitated with Grb2, we asked whether Spry proteins could inhibit the formation of Grb2-Sos complexes and/or the recruitment of Sos to the activated receptor. Under our conditions Grb2-Sos complexes were readily detected after FGF stimulation (Fig. 7C, bottom) and such complexes were unaffected by the presence of Spry proteins, despite the fact that Spry2 (but not Spry1) could be found in Sos immunoprecipitates (Fig. 7C, middle right). In addition, a 90-kDa tyrosine-phosphorylated protein, previously documented by several groups to be the membrane-anchored adaptor FRS2/SNT-1 (29, 30, 33), was also co-immunoprecipitated with Sos (Fig. 7C, top). Thus, Spry proteins do not inhibit the formation of the SNT/Grb2/Sos complexes. This implies that signaling from the FGF receptor to Sos and the recruitment of Sos to the membrane is not blocked by Spry proteins. On close examination of Fig. 7C (middle), a subtle but reproducible change in Sos was noted. Upon FGF stimulation, Sos migrated through the gel at a slightly reduced mobility consistent with a previously described hyperphosphorylation (32). This alteration in Sos mobility was almost completely abolished in the presence of Spry1 and was reduced in the presence of Spry2. This may be another reflection of the loss of Erk1/2 MAP kinases or other kinase activities downstream of Ras in the presence of Spry.

**DISCUSSION**

In this study, we methodically examined the consequences of Spry expression on signaling by the RTK using a biochemical approach. We showed that mammalian Spry proteins like *Drosophila* Spry, are able to antagonize a wide range of RTK. The fact that Spry1 and Spry2 could inhibit both proliferation of NIH3T3 cells and differentiation of PC12 cells indicates that the inhibitory effect of mammalian Spry proteins could affect cell fate depending on the cellular context. Thus, similarly to the factors it antagonizes, Spry activity does not seem to be specific to a biological response, but rather depends on the cellular environment. Interestingly, Spry proteins affect not only the strength of the signal but also its duration. This is of importance, since the duration of Erk1/2 activation was shown to be critical for cell fate determination, such as PC12 differentiation (34).

Growth factors were proposed to regulate Spry activity at different levels. First, growth factors can stimulate the relocalization of Spry proteins from the cytoplasm to the inner membrane of the cell (11). Second, *spry* expression is up-regulated by the pathway it antagonizes, yielding a negative feedback loop. In agreement with this model, growth factor treatment of NIH3T3 cells induced an immediate up-regulation of *spry2* expression. Unexpectedly, a down-regulation of *spry1* expression, parallel to the up-regulation of *spry2* expression, was observed upon FGF or PDGF treatment. Similar results were recently obtained in endothelial cells (35). These data indicate that the different Spry genes are not uniformly regulated and suggest that the different family members may not always be functionally equivalent. It also corroborated the fact that in some tissues the expression patterns of the Spry family members do not overlap (8). Thus, the individual Spry genes may be regulated by specific combinations of factors to allow optimal control of signaling. The regulatory logic of *spry1* down-regulation in NIH3T3 cells is currently unclear, as our assays did not allow us to see any consistent differences be-
Growth Factor

RTK

SNT

PI3-K

GRB2

AKT

GAP

RAS

GSK3-β

RAF

MEK

ERK

ELK

FOS

Fig. 8. A model for the action of mammalian Spry. Spry proteins antagonize growth factor signaling by specifically inhibiting the Ras/Raf/MAP kinase pathway. Spry proteins act downstream of the recruitment of Sos to the activated RTK and prevent Ras activation, possibly by blocking Sos activity or stimulating Ras deactivation through a Ras GAP protein.

between Spry1 and Spry2 activities. This may reflect a functional redundancy due to overexpression, or could be real as Spry1 and Spry2 are the most similar members of the family. Therefore, it would be interesting to examine the activities of Spry3 and Spry4, as these proteins are more divergent in their N-terminal domains.

The fact that mammalian and Drosophila Spry inhibit signaling through multiple receptors suggested that intracellular machinery common to signaling by the RTK is being affected. In the presence of Spry proteins, transcription of c-fos in response to FGF or serum was decreased. Induction by FGF was more dramatically affected. It should be noted that growth factors induction of c-fos expression is mainly mediated by the Ras pathway, whereas serum induction is in part independent of Ras (reviewed in Ref. 24). Transient co-transfection assays performed with a NF-κB reporter gene showed that Spry inhibited the stimulation by FGF or PDGF, but not by tumor necrosis factor-α, indicating the specificity of Spry proteins toward the Ras pathway. However, Spry proteins do not globally affect RTK signaling, as we showed that they inhibit the Ras/Raf/MAP kinase pathway but not the PI 3-kinase pathway. Since many cell survival signals through RTK are mediated by the PI 3-kinase pathway (reviewed in Ref. 36), its maintenance correlates with the fact that Spry suppressed cell growth, but did not lead to an increase in apoptosis. The induction of Spry in response to RTK activation might be a way of selectively altering the read out of the growth factor signal and might be adaptive during inductive interactions in development. The inhibition of the MAP kinase pathway and maintenance of other pathways including that of PI 3-kinase might allow the target cells to cease proliferation, yet maintain their viability.

Genetic analysis and in vitro assays suggested that Drosophila Spry was an inhibitor of the Ras pathway but the molecular mechanism of the inhibitory effect remained controversial (3, 5). Our finding that the phosphorylation of Akt was still induced by PDGF in the presence of Spry implied that binding of the ligand to the RTK still allowed the generation of second messengers and that the kinase activity of the receptor remained intact. This notion was confirmed by the fact that Spry expression did not affect the ability of the FGF receptor to mediate phosphorylation of one of its major substrates, FRS2/SNT-1. In contrast, we showed that Spry1 and Spry2 inhibited the activation of Ras, Raf-1, Mek1/2, and Erk1/2. The binding of Raf-1 to wild type, but not constitutively activated Ras, was reduced upon Spry expression. Furthermore, Spry proteins could not be shown to physically interact with Ras or Raf-1. Therefore, we propose that Spry proteins prevent the formation, or maintenance, of activated GTP-Ras. Since the kinase activity of RTK is not affected by the presence of Spry, our data indicate that Spry uncouples RTK from the chain of events that lead to the activation of Ras. Our model supports one of the genetic analysis performed in Drosophila (3) which showed that halving the dose of spry increased signaling by an activated RTK but not an activated Ras and thus indicates the functional conservation of the Spry family of proteins.

The exact mechanism of Spry action at the RTK/Ras interface needs to be clarified. Our experiments restrict Spry activity to a very narrow window. We found that Spry2 could form a complex in vivo with the adapter Grb2, which is also a property of the ancestral Drosophila protein (3), and Sos. This potentially could have prevented the coupling of the receptor to Sos. However, we showed that Spry2 did not impair the formation of the SNT-Grb2-Sos complex upon FGF stimulation. In addition, Spry1 did not immunoprecipitate with Grb2 or Sos. Together, this argues that Spry/Grb2 interaction is neither conserved, nor essential for Spry inhibitory effect. Our results indicate that Spry proteins do not block Ras activation by preventing Sos recruitment to the activated receptor as previously proposed (3). Therefore, Spry proteins potentially act downstream of the recruitment of the SNT-Grb2-Sos complex. Two possible modes of action may be proposed (Fig. 8). First, Spry proteins may inhibit the nucleotide exchange factor activity of Sos or limit its access to Ras. Second, Spry proteins may recruit a Ras GTPase activating protein (GAP) to the cell membrane or stimulate its activity to deactivate Ras. Neither Spry1 nor Spry2 could be demonstrated to bind to Ras GAP, which tends to argue against the deactivation model. In contrast, Casi et al. (3) found that Drosophila Spry could bind in vivo to the fly counterpart of Ras GAP. However, neither the ability of Drosophila Spry to bind in vivo to the fly Grb2 and Ras GAP, nor the functional consequences of these bindings have been addressed yet and may not be directly linked to its inhibitory effect.

As restriction of signaling is critical for proper patterning throughout development, the dual role played by Spry proteins in proliferation and differentiation raises the possibility that Spry malfunctions are responsible for developmental defects, as well as neoplasia. Our isolation of spry1 as a gene downstream of the WT1 tumor suppressor is consistent with this notion. Future studies should reveal the detailed molecular mechanism of the inhibitory effects of Spry as well as a possible role for these proteins in development and diseases.

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Functional Characterization of Mammalian Sprouty
