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Sprouty regulates cell migration by inhibiting the activation of Rac1 GTPase

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Abstract

Sprouty (SPRY) protein negatively modulates fibroblast growth factor and epidermal growth factor actions. We showed that human SPRY2 inhibits cell growth and migration in response to serum and several growth factors. Using rat intestinal epithelial (IEC-6) cells, we investigated the involvement of the Rho family of GTPases, RhoA, Rac1, and cdc42 in SPRY2-mediated inhibition of cell migration and proliferation. The ability of TAT-tagged SPRY2 to inhibit proliferation and migration of IEC-6 cells transfected with constitutively active mutants of RhoA(G14V), Rac1(G12V), and cdc42 (F28L) was determined. Constitutively active RhoA(G14V), Rac1(G12V), or cdc42(F28L) did not protect cells from the anti-proliferative actions of TAT-SPRY2. The ability of TAT-hSPRY2 to inhibit migration was not altered by of RhoA(G14V) and cdc42(F28L). However, Rac1(G12V) obliterated the ability of SPRY2 to inhibit cell autonomous or serum-induced migration. Also, the activation of endogenous Rac1 was attenuated by TAT-SPRY2. Thus, SPRY2 mediates its anti-migratory actions by inhibiting Rac1 activation.

Keywords: Cell motility; Intestinal epithelial cells; Rho GTPases; Cell proliferation

In the *Drosophila*, several proteins have been reported to modulate the activity of receptor tyrosine kinases [1]. Among these, Sprouty (SPRY) was discovered as a regulator of fibroblast growth factor (FGF) signaling [2]. In *Drosophila*, FGF is important in tracheal morphogenesis [3] and mutation in the *spry* gene resulted in increased tracheal branching [2]. Studies have subsequently reported that SPRY also modulates the actions of epidermal growth factor (EGF) in *Drosophila* [4–6] and therefore, SPRY has been claimed to be a general modulator of receptor protein tyrosine kinases.

With the cloning of four isoforms of mammalian SPRY proteins, SPRY1 to SPRY4 [2,7–9], there has been a tremendous interest in the biological actions of SPRY proteins and the mechanisms by which these proteins exert their effects. To this end, studies have demonstrated that SPRY1 [10], SPRY2 [11], and SPRY4 [12] inhibit serum or growth factor-stimulated cell migration and proliferation. Concerning the mechanisms involved in the actions of SPRY proteins, studies have shown that SPRY1 and SPRY2 inhibit the activation of the Erk pathway in response to some growth factors such as FGF and vascular endothelial-derived growth factor (VEGF) [10,12,13]. Decreased activation of Ras [4,12] or Raf [13] has been reported to be responsible for the inhibition of Erk activation. Notably, however, although SPRY1 and SPRY2 inhibit the proliferation and migration of cells in response to EGF [10,11], the

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activation of Erk by this growth factor is not altered by SPRY2 [10] (Yigzaw and Patel, unpublished observations). If anything, EGF-mediated Erk activation may be slightly enhanced by SPRY2 [14]. Therefore, it would appear that multiple mechanisms might be involved in mediating the anti-proliferative and anti-migratory actions of SPRY.

SPRY was originally thought to be secreted from cells [2]. However, later studies have shown that the protein is intracellular [4,11,15]. Human SPRY2 co-localizes with tubulin in the microtubular assembly, and activation of cells by growth factors results in the translocation of SPRY2 to membrane ruffles [11,15]. We have shown that if the translocation of SPRY2 to membrane ruffles is disrupted by deletion of its translocation domain, then the protein loses its anti-proliferative, and anti-migratory activities [11]. These findings suggested that one of the sites of SPRY2 action might be at the membrane ruffles. Upon activation, the Rho family of GTPases, Rac1, cdc42, and RhoA is also localized in membrane ruffles [16–18] and Rac1 has been shown to regulate growth factor induced membrane ruffling [19]. Moreover, the Rho family GTPases play a profound role in regulating cytoskeletal reorganization, migration, and proliferation [20–24]. Therefore, we postulated that one of the mechanisms by which SPRY modulates cellular migration and/or proliferation probably involves the modulation of activity of RhoA, Rac1, or cdc42. Using the wound-healing (scratch wound) assay, we demonstrate in this communication that wounding or scratch-elicited activation of Rac1, but not cdc42, in rat intestinal epithelial (IEC6) cells is inhibited by SPRY2 and that this is accompanied by inhibition of cell migration. Furthermore, we show that expression of constitutively active Rac1 rescues cells from the anti-migratory actions of SPRY2. None of the active forms of the Rho GTPases protected against the anti-proliferative actions of SPRY2. Thus, we conclude that SPRY2 inhibits cell migration by decreasing Rac1 activation.

Materials and methods

Cell culture. Rat intestinal epithelial (IEC6) cells over-expressing constitutively active Rac1, and Cdc42 in pMX-IRES-GFP were generated by transfecting the cells with plasmids pMX-IRES-GFP-G12V-Rac1 and pMX-IRES-GFP-F28L-Cdc42, respectively, as described previously [25]. Control cells were transfected with the empty plasmid in pMX-IRES-GFP. PMX-IRES-GFP constructs to express the Rho family of G proteins were the generous gift of Dr. Yi Zheng (Cincinnati Children's Hospital). Cells expressing constitutively active G14V-RhoA were derived as previously described [26]. The IEC6 cells were grown in DMEM (Cambrex, Walkersville, MD), supplemented with gentamicin sulfate (50 μg/ml; Cambrex, Walkersville, MD), insulin (10 μg/ml; Gibco–Invitrogen, Grand Island, NY), and 5% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA) at 37 °C in an atmosphere of 10% CO_2 in air.

Rac1 and cdc42 activity assays. Escherichia coli cells (TOP 10) expressing the Rac1 interactive domain of human PAK1 (residues 51–135) fused to glutathione *S*-transferase (GST) or GST-WASP in the plasmid pGEX-2T [27] were obtained from Dr. Yi Zheng, Cincinnati Children's Hospital. The transformed TOP 10 cells expressing GST-PAK1 or GST-WASP were grown to log phase in LB at 37 °C and induced with 0.2–0.4 mM isopropyl-β-D-1-thio-galactopyranoside (IPTG) for 2 h at 30–32 °C. Bacteria were lysed in a buffer containing 50 mM Tris, pH 7.4, 0.1% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, and protease inhibitors (10 μg/ml each of aprotinin and leupeptin) and bound to glutathione–Sepharose (Amersham Biosciences, Piscataway, NJ) as described by Kranenburg et al. [28].

Confluent IEC6 cells in 10 cm dishes were treated with 10 μg/ml each of TAT-SPRY2 or TAT-GFP for 60 min at 37 °C. Fig. 1 shows that the efficiency of transducing IEC-6 cells with TAT-tagged proteins is nearly 100%; to visualize TAT-SPRY2, the protein was labeled with FITC as described previously [11]. After transduction with either TAT-GFP (control) or TAT-SPRY2 multiple, and equal number of scratches in the dishes were made with a metal lice-removal comb (LiceMeister). Forty-five minutes later, cells were washed twice with ice-cold PBS and lysed as described previously [28]. Active Rac1 and cdc42 were bound to GST-PAK1 and GST-WASP, respectively, immobilized on glutathione–Sepharose at 4 °C for 60 min, and washed three times with lysis buffer. Active Rac1 and cdc42 in the GST-PAK1 or GST-WASP pull downs, respectively, as well as total Rac1 or cdc42 in IEC6 cell lysates were analyzed by Western blotting using monoclonal anti-Rac1 and anti-cdc42 antibodies (BD Biosciences Pharmingen, Lexington, KY).

Cell migration. IEC6 cells were grown to confluence in 40 mm dishes and treated with 10 μg/ml each of TAT-GFP (control) or TAT-SPRY2 for 60 min prior to wounding the monolayer by scratching with a gel-loading pipette tip as described previously [26]. Immediately thereafter, the scratches were photographed with a digital camera (Nikon) attached to a microscope (Olympus). The same fields were also photographed after 5 h to monitor the migration of cells. Scratch widths (in arbitrary units) were measured using Adobe Photoshop software.

Cell proliferation. IEC6 cells (10,000 cells) were plated in 40 mm dishes and allowed to attach before treating with 10 μg/ml TAT-SPRY2 or TAT-GFP (control). Medium containing fresh TAT-tagged proteins was replaced every day. At the times indicated, cells were lifted with trypsin–EDTA and counted using a hemocytometer.

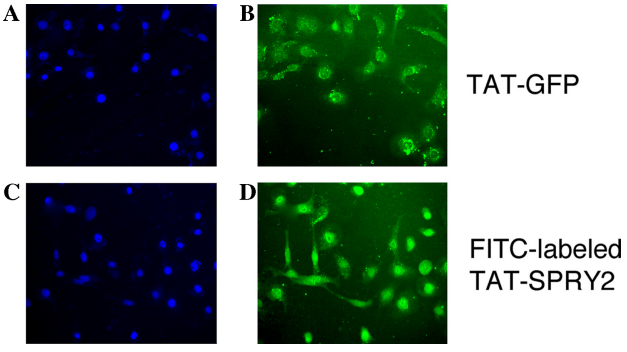

Fig. 1. Transduction of IEC6 cells with TAT-GFP and TAT-SPRY2. IEC6 cells (10^4 cells/35 mm dish) were grown in the presence of serum and exposed for 1 h at 37 °C to 10 μg/ml each of TAT-GFP or FITC-labeled TAT-SPRY2. Thereafter, the medium was removed and cells were fixed with 3.7% formaldehyde in PBS containing Ca^{2+} and Mg^{2+} for 5 min and 4′,6-diamidino-2-phenylindole (DAPI) containing mounting medium. The nuclei (A,C) and TAT-GFP (B) or FITC-labeled SPRY2 (D) were visualized using a fluorescence microscope. The images shown are at 200× magnification. Note that all TAT-tagged proteins are located in virtually all cells.

Results and discussion

Previous studies of Lim et al. [15] and from our laboratory [11] have demonstrated that when cells are activated by mitogens such as epidermal growth factor, the SPRY2 protein is translocated from microtubules to the membrane ruffles. Indeed, the deletion of the membrane translocation domain in SPRY2 obliterates the ability of the protein to inhibit cell migration and proliferation [11]. The formation of membrane ruffles, cell shape changes, lamellipodia formation, and cytoskeletal changes are events that precede cell migration and are attributed to the activation of small GTP binding proteins of the Rho family [29–31]. Moreover, the Rho family of GTPases has also been shown to modulate cellular proliferation [31]. Therefore, we postulated that the anti-proliferative and/or anti-migratory actions of SPRY2 are mediated by modulating the activation of one or more of the small GTP binding proteins, RhoA, Rac1, and cdc42. To address this hypothesis we used rat intestinal epithelial (IEC6) cells. These cells are particularly well suited for studies concerning migration since this process can be monitored well before the cells undergo proliferation. IEC-6 cells also do not express detectable amounts of SPRY2 (data not shown) and, therefore, are useful to study the function of exogenously introduced SPRY2. In our studies we used the approach of transducing cells with TAT-tagged SPRY2 or TAT-GFP (control). The TAT epitope on proteins permits their entry into cells [32,33] and we have previously shown that TAT-hSPRY behaves similar to transfected SPRY2 [11]. Fig. 1 shows that like HeLa cells [11], IEC-6 cells are very amenable to transduction with TAT-tagged proteins with almost a 100% transduction efficiency. To visualize TAT-SPRY2, the protein was labeled with FITC as described before [11]. Essentially, each cell shown by nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) (Figs. 1A and C) also shows the presence of TAT-GFP or FITC-labeled TAT-SPRY2 (Figs. 1B and D).

Initially, we examined the possibility that SPRY2 may modulate cell proliferation by attenuating the activation of Rac1, RhoA, or cdc42. For this purpose, IEC-6 cells were transfected with either plasmid alone (sham) or constructs expressing constitutively active (CA) forms of Rac1 (G12VRac1), CA-RhoA (G14VRhoA), or CA-cdc42 (F28L cdc42). The rationale in these experiments was that if SPRY2 mediated its anti-proliferative actions by attenuating the activation of one or more of the Rho GTPases, then the CA forms of these proteins should protect the cells against these effects. As shown in Fig. 2, the expression of CA forms of Rac1, RhoA, and cdc-42 augmented serum-stimulated cell proliferation above control levels. However, the transduction of TAT-SPRY2 inhibited the proliferation of IEC-6 cells irrespective of whether they expressed the CA-Rho proteins. These data demonstrate that activated Rho GTPases increase the proliferation of IEC6 cells and that the the anti-proliferative actions of SPRY2 are not attenuated by the activated forms of the Rho GTPases. These findings suggest that SPRY2 mediates its anti-proliferative actions either independent of modulation of the Rho GTPases or at locus/loci downstream of the Rho GTPases.

Next we investigated the possibility that SPRY2 mediates its anti-migratory actions by decreasing the activity of one or more of the Rho GTPases. IEC6 cells have been shown to migrate in a cell autonomous manner as well as in response to serum and growth factors. As shown in Fig. 3, in the presence of serum, IEC6 cells migrated faster than in the absence of serum. However, the transduction of TAT-SPRY2 into IEC6 cells inhibited the rate of cell migration both in the presence and absence of serum. Moreover, when IEC6 cells were

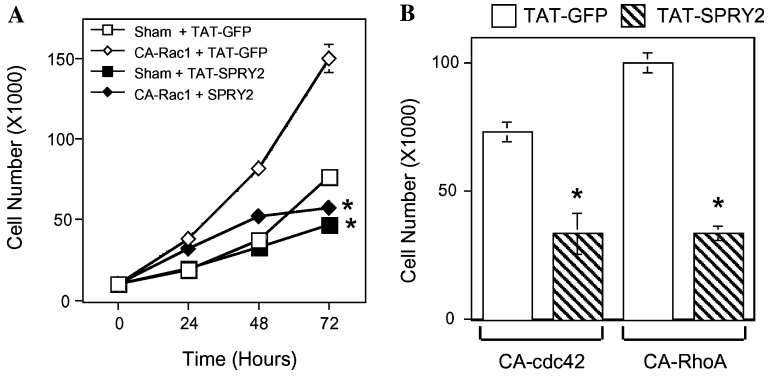

Fig. 2. Constitutively active (CA) Rac1, CA-RhoA, and CA-cdc42 do not protect against the anti-proliferative actions of SPRY2. IEC6 (10,000 cells) cells transfected with plasmid alone (Sham) or constitutively active Rac1 (A) or RhoA and cdc42 (B) were plated in 40 mm dishes and after 1 h exposed to 10 μg/ml each of TAT-SPRY2 or TAT-GFP (control-open symbols). The medium containing 5% fetal bovine serum and fresh TAT-tagged proteins was changed every 24 h. At 24, 48, and 72 h after plating, cells were lifted with trypsin/EDTA, and counted. For clarity, for cells expressing CA-cdc42 and CA-RhoA, the 72 h time point is shown separately in (B). Data presented are means ± SEM of at least three experiments. Wherever not visible, the error bars are within the symbol size. *$p < 0.002$ as compared to time control, Student's unpaired t test.

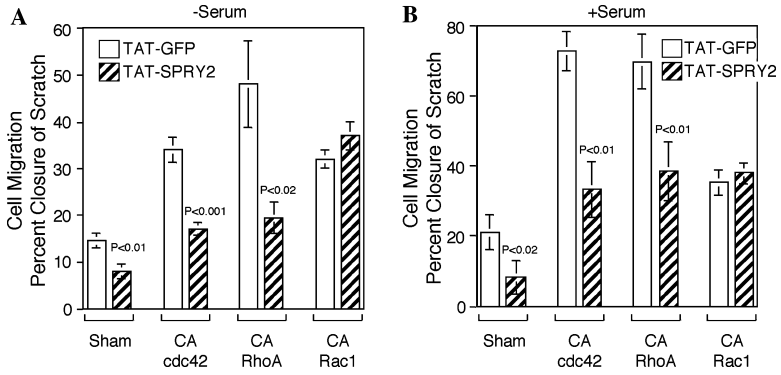

Fig. 3. Constitutively active (CA) Rac1, but not CA-RhoA or CA-cdc42, rescue IEC6 cells from the anti-migratory actions of SPRY2. IEC-6 cells were transfected with plasmid alone (Sham) or plasmid constructs to express constitutively active mutants of Rac1 (G12V) or RhoA (G14V) or the fast-cycling (F28L) mutant of cdc42 as described in Materials and methods. Confluent cells were incubated with or without serum overnight and treated with TAT-SPRY2 or TAT-GFP for 1 h prior to making a scratch with a pipette tip. Marked fields were photographed at time zero and 5 h after making the scratches and migration of cells was determined as percent closure of the scratch wound. The means ± SEM of at least three experiments are presented.

transfected to express CA-RhoA or the CA-cdc42, the rate of cell migration was elevated, indicating that these small GTP binding proteins can modulate the migration of IEC6 cells. However, SPRY2 transduction into cells expressing either the CA-RhoA or the CA-cdc42 inhibited cell migration, indicating that either SPRY2 exerted its anti-migratory actions below the level of cdc42 or RhoA or that SPRY2 inhibited a parallel pathway to RhoA and cdc42 that is also important for cell migration. The expression of the CA-Rac1 also stimulated cell migration (Fig. 3), demonstrating that Rac1 can also regulate this process. However, when cells were transfected to express constitutively active Rac1, the ability of SPRY2 to inhibit cell migration was obliterated (Fig. 3).

The data described above (Fig. 3) would suggest that the SPRY2 mediates its anti-migratory actions at a level above Rac1. If this contention was true, it would follow that the SPRY2 would regulate the activation of endogenous Rac1 in cells. To investigate this possibility, the modulation of endogenous Rac1 activation by SPRY2 in IEC6 cells was studied. Essentially, the activation of Rac1 in response to scratch wound was monitored in control (TAT-GFP) and TAT-SPRY2 transduced IEC6 cells. As shown in Fig. 4, scratch wounding increased Rac1 activation in control cells. This activation of Rac1 was markedly decreased in TAT-SPRY2 transduced cells. Interestingly, although scratch wounding activated cdc42, SPRY2 did not alter this activation. These findings (Fig. 4) are consistent with the ability of CA-Rac1, but not CA-cdc42, to rescue cells from the anti-migratory actions of SPRY2.

Since over-expression of CA-Rac1 increases growth of IEC6 cells (Fig. 2), it would appear that SPRY2 by decreasing Rac1 activation may decrease cell proliferation. However, unlike migration, the CA-Rac1 did not protect against the anti-proliferative actions of SPRY2 (Fig. 2), suggesting that in the case of the proliferative response, SPRY2 may also act at a locus/loci downstream of Rac1. The precise mechanisms involved in the anti-proliferative actions of SPRY2 are presently not known and form the subject of future investigations. Notably, however, studies in endothelial cells have shown that SPRY2 increases the cells in G1 phase of

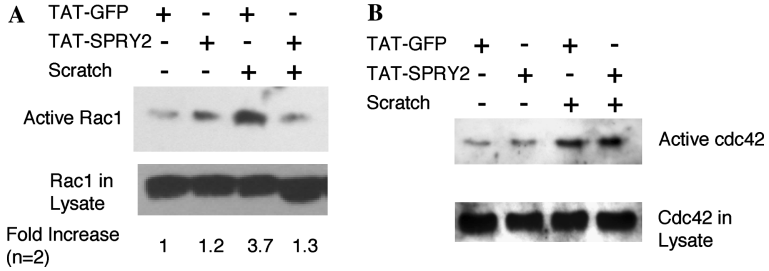

Fig. 4. Human Sprouty2 inhibits the activation of Rac1 but not cdc42. IEC6 cells grown to confluence were treated with TAT-SPRY2 for 1 h prior to making multiple and equal number of scratches as described in Materials and methods. The activation of Rac1 and cdc42 was monitored by pulling down the active G proteins with the substrate binding domain of PAK1 as a GST fusion protein and GST-WASP, respectively. The total amounts of Rac1 and cdc42 in the cell lysates used for pull-down of active G proteins were monitored by Western blotting with the appropriate antibodies. Representative experiments are shown. The values under each condition represent fold change in Rac1 activation over control.

the cell cycle [12]. We have also made a similar observation in HeLa cells (G. Ge and T.B. Patel, unpublished observations). Thus, it is likely that one or more critical steps in the G1 to S progression are inhibited by SPRY2.

Previously, we demonstrated that SPRY2 mediated increase in soluble protein tyrosine phosphatase 1B (PTP1B) contributes to its anti-migratory actions [34]. Indeed, a dominant negative mutant of PTP1B partially rescues cells from the anti-migratory actions of SPRY2 [34]. Moreover, the expression of SPRY2 decreases tyrosine phosphorylation of the PTP1B substrate p130Cas [34]. Because tyrosine phosphorylated p130Cas in complex with CrkII, DOCK180, and ELMO can act as a guanine nucleotide exchange factor for Rac1 [35–37], it is tempting to speculate that increase in soluble PTP1B activity by SPRY2 and the resultant decrease in p130Cas phosphorylation may be one mechanism by which SPRY2 attenuates the activation of Rac1. Alternatively, since SPRY2 binds to phosphatidylinositol 3,4-bisphosphate (PIP_2), at the cell membrane [38], it is possible that the conversion of PIP_2 to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) by phosphatidylinositol 3-kinase is impeded. The reduced amount of PIP_3 may then decrease activation of Rac1-specific guanine nucleotide exchange factor(s) such as TIAM1/2 that are activated by this phospholipid [30]. These and other possibilities form the subject of future investigations.

In sum, our data demonstrate that the activation of Rac1 is attenuated by SPRY2 and that CA-Rac1 can rescue cells from the anti-migratory actions of SPRY2. Concerning cell proliferation, since CA-Rho GTPases increased cell growth, and SPRY2 decreases Rac1 activation, CA-Rac1 would have been expected to protect against the anti-proliferative actions of SPRY2 if the only actions of SPRY2 were upstream of Rac1. The opposite finding (Fig. 2), however, suggests that SPRY2 may also act at locus/loci downstream of Rac1 to modulate cell proliferation. Thus, regulation of proliferation and migration by SPRY2 involve divergent pathways.

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