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srGAP3 promotes neurite outgrowth of dorsal root ganglion neurons by inactivating RAC1

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ABSTRACT

Objective: To explore effect of srGAP3 promotes neurite outgrowth of dorsal root ganglion neurons. **Methods:** In this study, expression of Slit1 was observed predominantly in the glia, while expression of Robo2 and srGAP3 was detected in sensory neurons of postnatal rat cultured dorsal root ganglion (DRG). Furthermore, upregulation of srGAP3 following sciatic nerve transection was detected by immunohistochemistry and Western blotting. **Results:** It was observed that inhibition of neurite outgrowth in cultured adult DRG neurons following treatment with anti-srGAP3 or anti-Robo2 was more effectively (1.5-fold higher) than that following treatment with an anti-BDNF positive control antibody. It demonstrated that srGAP3 interacted with Robo2 and Slit1 protein to decrease Rac1-GTP activity in cultured adult rat DRG neurons and the opposite effect on Rac1-GTP activity was detected by co-immunoprecipitation and immunoblotting analyses following treatment with anti-Robo2 or anti-srGAP3. These data demonstrated a role for srGAP3 in neurite outgrowth of DRG sensory neurons. **Conclusions:** Our observations suggest that srGAP3 promotes neurite outgrowth and filopodial growth cones by interacting with Robo2 to inactivate Rac1 in mammalian DRG neurons.

1. Introduction

Neuronal migration and axon guidance are known to be complex processes for which the underlying molecular mechanisms are gradually being elucidated. Slit protein has emerged as an important repulsive cue among a variety of extracellular guidance cues in the development of the central nervous system[1-3]. Functions of the Slit protein as a promoter of axonal branching[4] and as a guidance cue for neuronal migration have also been reported[5]. The roles of Slit protein are known to be dependent on interaction with the extracellular domain of the Roundabout (Robo) receptor[6].

The Rho GTPases, downstream of the Slit-Robo signaling pathway are key regulators of the actin cytoskeleton reorganization in eukaryotic cells and have been implicated in axon guidance[7]. These enzymes also regulate dendrite elaboration and neurite outgrowth[8-11]. Rho GTPases exist in two interconvertible forms: the active (GTP-bound) and inactive (GDP-bound) states, which are controlled by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). The ability to cycle between these two forms and to transmit signals to the downstream effector molecules allows Rho GTPases to act as signaling switches. GEFs and GAPs exhibit mutually antagonistic activity; GEFs catalyze nucleotide exchange for activation, while GAPs promote GTP hydrolysis, leading to inactivation[12]. Wong *et al*[13] identified a novel family of GAPs, designated the Slit-Robo GTPase activating proteins (srGAPs), consisting of three members: srGAP1, 2 and 3. srGAPs contain a Rho GAP domain that regulates the activity of Rho family GTPases

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and affects actin polymerization, as well as a SH3 domain that interacts with the CC3 motif of Robo[13,14].

In cultured mammalian cells, srGAP1 can bind to and decrease the level of active Cdc42 and RhoA, but not Rac1. Extracellular application of Slit to primary neurons increases the intracellular binding of Robo and srGAP1, and inhibits Cdc42 activity in a Robo- and srGAP-dependent manner[15-17]. Wong *et al*[13] reported that srGAP1 can transfer the repulsion signal of Slit by inactivating Cdc42 in neuronal migration. Moreover, a dose-dependent relationship between srGAP1-Robo interaction and Slit concentration has been demonstrated[18]. mRNA expression of both srGAP2 and srGAP3 has been shown in the dorsal root ganglia (DRG) during development[19]. We have reported in previous study that expresse level of both Robo2 and srGAP3 in adult rat DRGs is upregulated following axon injury[20]. Moreover, our in vitro studies observed that Robo2 may promote neurite outgrowth of cultured primary DRG neurons[21]. However, the involvement of srGAP in this process and the underlying mechanism of action remain to be established. Therefore, in present study, we investigated the influence of srGAP3 in neurite outgrowth and the underlying mechanism in cultured rat DRG neurons.

2. Materials and methods

2.1. Animals and surgery

Experiments were performed with the approval of the local animal ethics committee, in accordance with guidelines for animal experiments established by the Hainan Medical College and the Chinese Government nimal protection and management laws. Adult Sprague-Dawley (SD) rats [(250×20) g] were purchased from the Hunan Agricultural University (P.R. China). For transection, rats were anesthetized by intraperitoneal (ip.) injection of 10% chloral hydrate (0.4 g/kg) and fixed into a prostrate position. The right sciatic nerve at the mid-thigh was exposed under sterile conditions and transected 3 mm proximal to its division into the tibial and common peroneal nerves. Rats were sacrificed at various time-points (0, 1, 3, 7, and 14 days) post-surgery. Tissues collected included two sides of the L3-L4 DRG (along the sciatic nerve traced back to the DRG).

2.2. Primary neurons cultures

Primary sensory neurons were routinely cultured from the DRG of SD rats (aged 3-4 weeks). Cells from each rat were dispersed evenly on 10 poly-D-lysine- and laminin-coated 13 mm glass coverslips and cultured in a 24-well plate for 4 h at 37℃ under 5.0% CO_2 in a phenol-red-free neurobasal medium (Gibco, Carlsbad, CA, USA) with B-27 serum-free supplement, plus 7.5 pg/mL nerve growth factor (NGF), 200 μM GlutaMAX-1, and 1 g/mL cholesterol as low-density lipoproteins (Sigma-Aldrich, St. Louis, MO,USA) prior to the addition of different solvents or no addition. Cultured cells were fixed with 4% paraformaldehyde (PFA) prior to commencing experiments [22,23].

2.3. Immunocytochemical assay

To detect expression of srGAP3, untreated cultured neurons after being cultured for 48 h were stained with chicken polyclonal anti-class Ⅲ β-tubulin (1:1 000; Millipore, Bedford, MA, USA) and rabbit polyclonal anti-srGAP3 (1:200; Santa Cruz, CA, USA) antibodies. Secondary antibodies included: goat anti-rabbit IgG Cy3 conjugated affinity purified secondary antibody (1:300; Millipore), rabbit anti-chicken IgG FITC-conjugated affinity purified secondary antibody (1:100; Millipore). Negative controls were prepared using the same protocol without the addition of the primary antibodies.

Cultured primary neurons were pretreated with anti-srGAP3 antibody (20 μg/mL), anti-Robo2 antibody (15 μg/mL), or recombinant Slit1 (500 ng/mL). Anti-BDNF (50 ng/mL) was used as the positive control and normal sheep IgG (10 μg/mL) as negative control. Cultured cells after being cultured for 48 h were stained with anti-class Ⅲ β-tubulin antibody to visualize axons as previously described[24].

To visualize neurite length, the cultured DRG neurons were stained with anti-class Ⅲ β-tubulin and rabbit polyclonal anti- srGAP3 antibodies at 24, 36, and 48 h following pretreatment with anti-srGAP3 antibody (20 μg/mL).

To investigate srGAP3 and Robo2 co-expression in adult rat DRG neurons, HEK293 cells (Invitrogen, Carlsbad, CA, USA) were stained with rabbit polyclonal anti-srGAP3 and goat polyclonal anti-Robo2 (1:200; Santa Cruz). Secondary antibodies included FITC-conjugated donkey polyclonal anti-goat IgG (H&L) (1:1 000; Abcam, Hong Kong) and goat anti-rabbit IgG Cy3 conjugated affinity purified secondary antibody.

To detect expression of Rac1 in rat DRG neurons, cultured explants were pretreated with anti-srGAP3 antibody (20 μg/mL), anti-Robo2 antibody (15 μg/mL), or recombinant Slit1 (500 ng/mL). Untreated explants served as controls (con). Adult rat DRGs neurons after being cultured for 48 h were then stained with rabbit polyclonal anti-Rac1 antibody (1:100; Abcam). FITC-conjugated donkey polyclonal anti-rabbit IgG (1:300; Abcam) were used as the secondary antibody.

2.4. Immunohistochemcal assay

The L3 and L4 DRGs were removed from adult rats for immunostaining of DRG cells. Tissues were then embedded

in a fresh-frozen, optimal cutting temperature (OCT) compound and cut into 15 μm transverse DRG (n=6 for each time point) sections on a cryostat. Sections were then fixed with acetone, blocked in 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), and incubated with primary antibodies at 4℃ for 24 h. The primary antibodies were: mouse polyclonal anti-Slit1 (1:200; Abcam), rabbit polyclonal anti-srGAP3, goat polyclonal anti-Robo2 and mouse polyclonal anti-GFAP (1:200; Abcam). After rinsing in PBS, the sections were incubated with secondary antibodies at room temperature (RT) for 2 h. Secondary antibodies were: FITC-conjugated goat polyclonal anti-mouse IgG (1:300; Abcam), donkey polyclonal anti-goat IgG (H&L) and Cy3-conjugated affinity purified anti-rabbit IgG. Images of eight sections per animal were obtained by fluorescence microscopy (Olympus, Tokyo, Japan, BX51). Standard staining protocols were used[25,26].

2.5. Western blot analysis

L3-L4 DRGs were removed from adult rats and lysed in a buffer containing 100 mM HEPES (pH 7.4), 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 10 mg/mL pepstain and 10 mg/mL leupetin. The concentration of protein in the homogenate was determined using the BCA reagent (Pierce, France). Protein lysates (50 mg) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking in 1% BSA in washing buffer containing 10 mM Tris (pH 7.5),150 mM NaCl and 0.05% Tween-20, membranes were incubated overnight at 4 ℃ with mouse polyclonal anti-Slit1 (1:1 000; Abcam), rabbit polyclonal anti-srGAP3 (1:2 000; Santa Cruz), goat polyclonal anti-Robo2 (1:2 000; Santa Cruz) and chicken polyclonal anti-β-class Ⅲ tubulin (1:2 000; Millipore) and mouse monoclonal anti-β-actin (HRP) (1:20 000; Abcam). After washing three times with TBS-T (50 mM Tris, 150 mM NaCl, 0.1% Tween-20), membranes were incubated for 2 h with chicken anti-mouse IgG-HRP (1:2 000; Santa Cruz), mouse anti-rabbit IgG-HRP (1:5 000; Santa Cruz), donkey anti-chicken IgY (H&L) (HRP) secondary antibody (1:2 500; Abcam), mouse anti-goat IgG-HRP (1:1 000; Santa Cruz) at RT. Signals were detected using the ECL kit (GE Healthcare Life Sciences, Connecticut, USA). The intensity of protein bands was determined using the NIH Image 1.63 software (National Institutes of Health, Bethesda, MD, USA).

2.6. Pull-down assay for activation of Rac1 and Cdc42

The srGAP3-dependent activation of Rac1 and Cdc42 was assessed using a pull-down assay kit according to manufacturer instructions (NewEast Biosciences, USA). DRG neurons from SD rats were cultured and treated with 30 ng/mL anti-srGAP3 (0, 10, 30 and 60 min) and subsequently homogenized and lysed with RIPA buffer containing protease inhibitors (Roche Applied Science, Indianapolis, USA). One aliquot was used for immunoblot (IB) analysis and the second aliquot was incubated with GTP-Rac1 (1:1 000; Abcam) or GTP-Cdc42 (1:1 000; CST, Boston, USA) antibodies, bound with protein A/G agarose beads for 1 h at 4℃, precipitated and subjected to SDS-PAGE. Proteins were detected using anti-Rac1 or anti-Cdc42 antibodies. GTP-activated levels were normalized to the corresponding total Rac1 levels by densitometric analysis using ImageJ software. The data are expressed as a percentage of the signals obtained at 10, 30 and 60 min in comparison with the signal detected at 0 min[27].

2.7. Image analysis

Imaging was performed by fluorescence microscopy (Olympus BX51). Image capture parameters were initially established and then used for all subsequent images. Five random images at a magnification of 20× were collected, with non-overlapping images obtained from the L4 and L5 DRG from each of five animals per condition (25 images in total). For each image, all neurons with visible nuclei were counted first. The background was defined by sampling the image pixel intensity from neurons with larger diameters that were not labeled by any antibodies. All neurons with visible nuclei displaying fluorescent signals two standard deviations above background were counted as moderately immunoreactive, while images with fluorescent signals three standard deviations above background were counted as intensely immunoreactive. Neurons with fluorescence signals less than two standard deviations above the background were considered to be negatively immunoreactive. EZ-CI (Leica, Germany) image analysis software was used for these studies.

Images of the cultured explants were acquired from randomly selected fields (n=8-12) by fluorescence microscopy (Olympus BX51). The length of the longest neurite of 80 to 120 neurons per condition was determined using ImageJ software[28].

2.8. Statistical analysis

All data were analyzed using SPSS 13.0. The number of positively immunoreactive neurons and neurite lengths detected are presented as the mean±SEM. Statistical analyses were conducted using the Student's t-test, one-way ANOVA, and the Dunnett t-test. P<0.05 was considered to indicate statistical significance.

3. Results

3.1. Expression of Slit-Robo pathway signaling molecules in injured DRG

We have previously observed that srGAP1 is mainly expressed in the glia, while srGAP2 is not expressed, and srGAP3 is expressed in neurons in adult mammal DRG; therefore, this study was focused on the expression of srGAP3 in adult rat neurons. Using double immunostaining for Slit1 and srGAP3, GFAP and srGAP3 as well as Robo2 and srGAP3, results showed that Slit1 was expressed strongly in satellite glia cells (SGC) and weakly in neurons (Figure 1A, C), srGAP3 was expressed in neurons (Figure 1B, E, H) and srGAP3 and Robo2 were co-expressed in a subset of DRG neurons (Figure 1G-I). Furthermore, srGAP3 was expressed in cultured primary neurons, including its processes (Figure 2). Subsequently, using double immunostaining, showed co-expression of Slit1 and glutamine synthetase (GS, a satellite cell marker) in DRG (SGC) (date not show). Western blot analysis was used to determine the protein expression levels of Slit1, Robo2 and srGAP3 in DRG tissues. Slit1 was detected as a single band at 168 kD, Robo2 at 145 kD and srGAP3 at 160 kD, β-tubulin Ⅲ as an internal loading control, a single band at 80 kD, (Figure 1J). Furthermore, some small molecular weight bands were strongly detected as Robo2 and srGAP3. These bands are likely to be Robo2 and srGAP3 cleavage products[29].

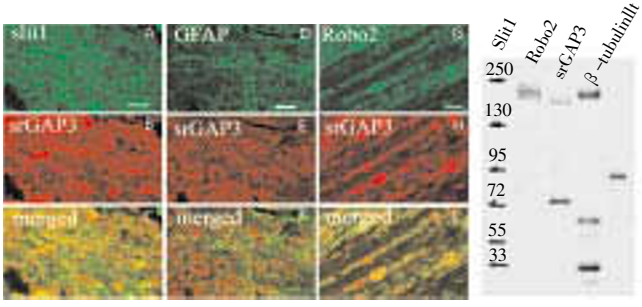

Figure 1. Slit1, Robo2, srGAP3 expression in adult rat DRG.
Double-immunofluorescent staining of Slit1 or srGAP3 and GFAP or Robo2 in injured adult rat DRG at 3 days post-surgery.
(A-C) double labeling of Slit1 (green) and srGAP3 (red) in DRG. Note: Slit1 is expressed in small satellite-like cellular profiles and neurons. (D-F) double-immunofluorescent staining of srGAP3 and the glial marker, GFAP. SrGAP3 is expressed in neurons (red) around the glia (green). (G-I) DGR neurons co-express srGAP3 (red) and Robo2 (green). Note: Robo2 showed strong positive expression in the membrane of DRG neurons. Scale bar = 50 μm. Right panel, (J) Western blot using affinity purified anti-Slit1, anti-Robo2, anti-srGAP3 and anti-β-class Ⅲ tubulin antibodies. The expected molecular weight of Slit1 (Lane 1), Robo2 (Lane 2), srGAP3 (Lane 3) and β-class Ⅲ tubulin (Lane 4) are 168, 145, 160 and 80 kD, respectively. Proteins was detected using standard techniques. Note: The smaller strong bands observed in this blot are likely to represent Robo2 and srGAP3 cleavage products.

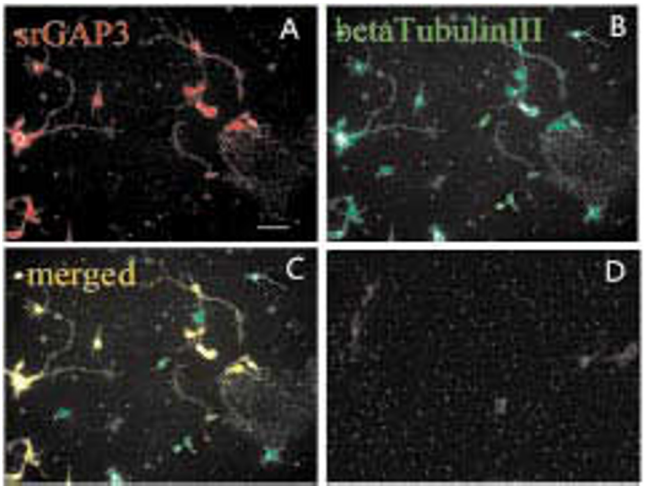

Figure 2. Representative double-immunofluorescent staining of srGAP3, β-class Ⅲ tubulin in cultured adult rat DRG neurons.
(A) srGAP3 is expressed in cultured adult rat DRG neurons. (B) β-class Ⅲ tubulin is expressed in adult rat cultured DRG neurons. (C) A and B (merged) show srGAP3 co-localizes with β-class Ⅲ tubulin. (D) Staining in the absence of the primary antibody. Scale bar = 50 μm.

To investigate the effect of axon injury on srGAP3 expression in DRG, we determined the protein expression levels of srGAP3 with snit-srGAP3 antibody immunostaining (Figure 3A-F) and western blotting analyses following the sciatic nerve transection at day 0, 1, 3, 7, 14 (Figure 3G). β-actin was used as an internal loading control in western blotting analysis. The results showed that expression level of srGAP3 was increased at day 7 post-surgery, which persisted to day 14. Compared to the collateral side at day 0 or 1, the ratio of srGAP3 immune-positive (the number of intense srGAP3 immune-positive neurons/total number of srGAP3 immune-positive neurons) was significantly increased ($P<0.01$) (Figure 3H).

3.2. SrGAP3 promotes neurite outgrowth in cultured DRG neurons

In this assay, cultured primary adult DRG neurons were treated with different factors

To demonstrate an influence of srGAP3 on cultured postnatal rat DRG neurons, cultured neurons were pretreated with different factors and the length of processes was measured following anti-class Ⅲ β-tubulin immunostaining (Figure 4A-E). The neurite length was significantly reduced in presence of anti-srGAP3 antibody (76.71 μm) and anti-Robo2 antibody (73.68 μm) or anti-BDNF antibody (as the positive control, 96. 12 μm). In contrast, the length was increased after pretreatment for

48 h with recombinant mouse Slit1 (re-hu-Slit1) protein (156.37 μm) compared to the IgG control (104.53 μm) (P< 0.05) (Figure 4F). These results suggest that Slit1, Robo2 and srGAP3 promote neurite outgrowth in DRG neurons.

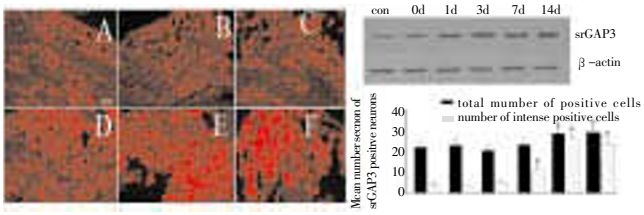

Figure 3. srGAP3 is upregulated by axonal injury.
Immunofluorescence of srGAP3 in ipsilateral relative to contralateral DRG following sciatic nerve transection in animals surviving from day 0 through day 14. (B-F) The pattern of immunolabeling remains largely unchanged in the transected DRG relative to the control, involving neuronal profiles. (G) Representative Western blot image of srGAP3 following sciatic nerve transection in animals surviving from day 0 through day 14. However Densitometry indicates an increase in the total number of immunoreactive cells (mean × SEM) as well as the number of cells with intense labeling (H) relative to control. * indicate a statistically significant difference of P<0.01 compared to normal DRG; + indicates a statistically significant difference of P<0.01 compared to 1 and 3 days. Con: normal DRG. One-way ANOVA and Dunnett t-tests, n = 5/time point. The scale bar = 50 μm for image panels.

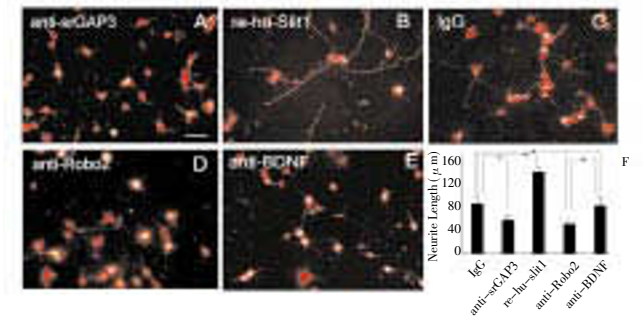

Figure 4. srGAP3 promotes neurites elongation of cultured DRG neurons.
(A-E) Representative images of dissociated cultured DRG neurons immunostained for β-class Ⅲ tubulin. Goat IgG treated neurons (C) is compared with neurons pretreated with anti-srGAP3 antibody (A), or anti-Robo2 antibody (D), or recombinant Slit1 (500 ng/mL) (B), pretreated with anti-BDNF as the positive control (E). Quantitative analysis of neurite length for 100 dissociated neurons for each treatment group. F shows the length of neuron processes following different treatment. Values shown are the means ± SEM from three independent experiments. One-way ANOVA and Dunnett t-tests, n= 5/time point. * compared to each other, P<0.05. The scale bar=50 μm for image panels.

Furthermore, double-staining with anti-srGAP3 and anti-class Ⅲ β-tubulin following anti-srGAP3 treatment for 24, 36 and 48 h in cultured DRG neurons (Figure 5A-I), the results showed that the mean value of neurite lengths of srGAP3 immune-positive cells (43.14, 57.98 and 64.32 μm, respectively) was less than those of total cells (β-tubulin Ⅲ immune-positive cells) (112.34, 160.67 and 186.64 μm, respectively) at all three time-points (Figure 5J) (P<0.05). These results indicate that anti-srGAP3 antibody acted only on srGAP3 immune-positive neurons, thus implying that srGAP3 maintains neurite outgrowth of adult mammalian sensory neurons.

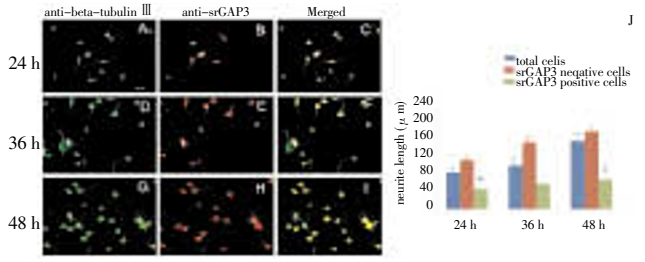

Figure 5. srGAP3 promotes neurites outgrowth of srGAP3 immune-positive neurons.
(A-I) Representative images of dissociated cultured DRG neurons immunostained for β-class Ⅲ tubulin and srGAP3.The neurons were pretreated with anti-srGAP3 antibody. Panels show immunostaining with: β-class Ⅲ tubulin (left), srGAP3 (middle) and merged images of (right) at 24 h (upper), 36 h (middle) and 48 h (lower) following pretreatment with anti-srGAP3 antibody. Note: srGAP3 immune-positive neurons show shorter neurites compared with srGAP3 negative neurons. (J) Quantitative analysis of neurite length for 100 dissociated neurons for each time point, the lengths of srGAP3-positive neurons processes are shorter than those of srGAP3-negative neurons. Data are the means ± SEM from three independent experiments, *compared with total cells or srGAP3-negative cells P< 0.05. Scale bar = 50 μm for image panels.

3.3. Anti-srGAP3 antibody increased Rac1 not Cdc42 activity

As downstream signaling molecules in the Slit-Robo pathway, Rac1 and Cdc42 provide the necessary integration sites for the complex regulation of growth cone motility, cell division, and actin organization in neuronal growth. Therefore, the involvement of Rac1 and Cdc42 in srGAP3-mediated neurite outgrowth was then investigated. Rac1 and Cdc42 activity was detected using specific GTP-Rac1 and GTP-Cdc42 antisera. As shown in Figure 6A, anti-srGAP3 treatment caused a time-dependent increase in GTP-Rac1 immunoreactivity in cultured DRG cells. After treatment with anti-srGAP3 for 30 and 60 min, GTP-Rac1 immunoreactivity increased (2.19 × 0.32)-fold and (2.56 × 0.41)-fold, respectively (Figure 6B), whereas the anti-GTP Cdc42 antibody immunoreactivity was similar at both time-points (Figure 6B).

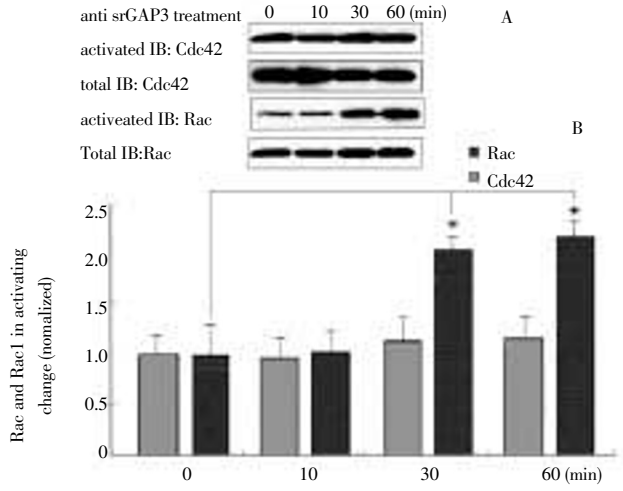

Figure 6. Anti-srGAP3 antibody increased Rac1 activity in cultured DRG neurons.

(A) Activated and total levels of Rac or Cdc42 were detected by immunoblotting (IB) after anti-srGAP3 antibody (0, 10, 30, 60 min) treatment. (B) 30 and 60 min exposure to anti-srGAP3 antibody caused a significant increase in activated Rac1 normalized to total Rac.*P < 0.01, compared to untreated neurons (con); However, exposure to anti-srGAP3 antibody did not alter activated Cdc42 levels normalized to total Cdc42.

3.4. Slit1-Robo2 signaling depends on decreased Rac1 activity to promote neurite outgrowth

Rac1activity was next detected using specific GTP-Rac1 antisera after re-hu-Slit1, anti-Robo2 or anti-srGAP3 antibody treatment. As shown in Figures 7A and B, compared to the untreated control (con), Slit1 treatment resulted in decreased GTP-Rac1 immunoreactivity, while anti-Robo2 or anti-srGAP3 antibody treatment resulted in increased GTP-Rac1 immunoreactivity. The GTP-Rac1 normalized activity increased (2.45 × 0.34)-fold and (2.68 × 0.56)-fold following anti-srGAP3 or anti-Robo2 treatment.

The ratio of weakly GTP-Rac1 immune-positive cells was determined using anti- GTP-Rac1 immunostaining. The ratio following anti-srGAP3 and anti-Robo2 antibody treatment was significantly lower than that detected following re-hu-slit1 protein treatment. Compared to the untreated control group, the ratio of weakly GTP-Rac1 immune-positive cells following Slit1 treatment increased by (45.56×7.09)%, that of anti-srGAP3 treatment decreased by (24.37×6.12)%, and that of anti-Robo2 treatment decreased by (18.86×6.21)% (Figure 7C, D). These results suggest that Slit1-Robo2 signaling depends on decreased Rac1 activity to promote neurite outgrowth.

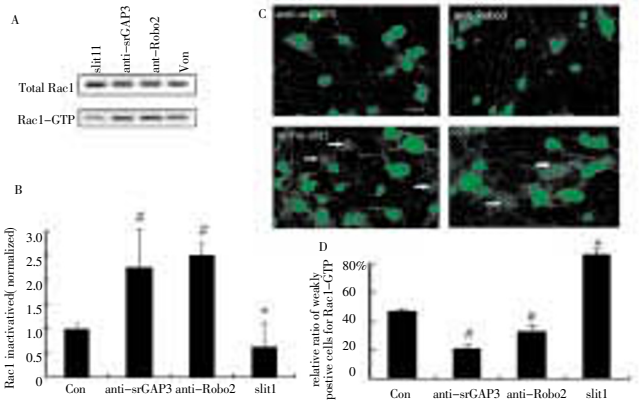

Figure 7. SrGAP3 promotes neurite growth through inactivation of Rac1

(A) Rac1- GTP was analyzed by Western blotting of cultured DRG neurons following pretreatment with anti-srGAP3 antibody, anti-Robo2 antibody or re-hu-Slit protein. Untreated DRG neurons were used as controls (con). (B) Exposure of cells exposured to re-hu-slit1 protein caused significant downregulation of Rac1-GTP normalized to total Rac1, while exposure to anti-srGAP3 or anti-Robo2 antibody caused significant upregulation of Rac1-GTP. # and *P<0.01, compared to untreated neurons. (C) RAC1-GTP immunolabeling of cultured DRG neurons treated with anti-srGAP3 antibody, anti-Robo2 antibody or re-hu-Slit protein. Untreated DRG neurons were used as controls (con). Arrows indicate weak Rac1 immunostaining. Scale bar = 50 μm for image panels. (D) Statistical analyses of cultured DRG neurons shows that the number of weak Rac1-GTP immunostaining positive cells detected following anti-srGAP3 or anti-Robo2 antibody treatment is significantly higher than the that detected following Slit1 protein treatment, # and * P<0.01, compared to the untreated group (con).

4. Discussion

Growth of neuronal axons and dendrites depends on the dynamics of the cytoskeleton, which is regulated by soluble extracellular cues, the matrix, cell surface receptors and intracellular signals[30]. There are several positive and negative regulators that guide primary axonal growth cones, including Slit and its receptor Robo, which have been found to be expressed in the brain at different developmental stages[14,19,31]. In a study by Wang *et al*[4], the amino-terminal fragment of Slit2 was shown to function as a sensory axon elongation- and branch-promotion factor in NGF-responsive sensory neurons. These results suggested that Slit proteins have the potential to participate in regulating the formation or extension of axon collaterals during mammalian development. In our previous study, soluble Slit1 protein was found to promote cultured DRG neurite outgrowth and elongation. In contrast, a recombinant human Robo2/Fc chimera inhibited neurite outgrowth and elongation.

However, the downstream molecular mechanism underlying the role of the Slit-Robo signaling pathway in neurite outgrowth remains unclear. The srGAPs were identified as a family of GAPs that interact with the intracellular domain of Robo[13]. We hypothesized that srGAPs are highly expressed in DRG neurons, and act as a positive regulators of neurite growth via interaction with Robo and inactive Rac or Cdc42. Expression of srGAP3 was upregulated following axotomy, which is consistent with the expression pattern previously described for the transmembrane receptors, Robo1 and Robo2[32]. Interestingly, Madura *et al*[33] reported upregulation of srGAP expression in the facial nucleus after transection of the facial nerve in adult rats. The combination of these results suggests that the putative function of srGAP3 may be related to regeneration of the peripheral nerves[33,34]. Treatment of cultured neurons with an anti-srGAP3 antibody to block the endogenous srGAP3 resulted in decreased neurite length of cultured DRG sensory neurons. An identical effect was observed following treatment with an anti-Robo2 antibody. To further confirm the inhibitory effect of the anti-srGAP3 antibody on neurite growth, an anti-BDNF antibody was used as a positive control for comparison. Both antibodies inhibited neurite growth, although the anti-srGAP3 antibody was 1.5-fold more potent than the anti-BDNF antibody. Furthermore, double immunostaining of β-class Ⅲ tubulin and srGAP3 was employed to determine the population of anti-srGAP3 antibody reactive cells involved in this role, Significantly, we observed that the anti-srGAP3 antibody exerted its effect only on srGAP3 immune-positive neurons, thus indicating that the anti-srGAP3 antibody specifically antagonizes srGAP3 resulting in inhibition of neurite growth. Therefore, we postulate that srGAP3 interacts with Robo2 to promote neurite outgrowth of DRG sensory neurons.

Reports suggest that the three mammalian members of the Slit-Robo GAP family (srGAP1-srGAP3) are involved in different intracellular signaling cascades. Although srGAP1 represents a GAP protein for Cdc42, both srGAP2 and srGAP3 have been shown to downregulate Rac signaling[13,31,35,36]. Furthermore, srGAP2 has been shown to interact with Wiskott-Aldrich syndrome protein (WASP), whereas srGAP3 binds to the related Wiskott-Aldrich syndrome protein family member 1(WAVE1) protein. This suggests that the three srGAP members link Slit-Robo to different signaling cascades, which is supported by the observation that they are embryonically expressed in distinct subsets of neural tissues[19]. srGAPs share a common molecular architecture, consisting of an N-terminal F-BAR domain, a centrally located GAP and a Src-homology 3 (SH3) domain[13,31,35,36]. The SH3 domain of all three members has been shown to interact with the intracellular part of the Robo1 receptor[13]. Robo1 and its ligand, Slit, are involved in a variety of developmental processes including neuronal migration and steering of axonal projections[1,37,38]. srGAP3 (also known as WRP or MEGAP) regulates Rac activity[36] and is implicated in severe X-linked mental retardation[31]. Yao *et al*[34] showed that srGAP3 co-localizes with Robo1 in the ventral and lateral funiculus and with Robo2 in the lateral funiculus of the developing spinal cord. Despite some controversy regarding srGAP3 distribution and release [19,34], srGAP3 has been detected in DRG neurons by Western blot and immunohistochemical analyses[20]. These studies strongly indicate that endogenous srGAP3 has physiological and pathological effects on DRG axonal growth. Here, we demonstrated that srGAP3 interacts with Robo2, through co-immunoprecipitation experiments with HEK293 cells. The demonstration of Robo2 and srGAP3 co-expression in the same population of DRG neurons and Slit1 in DRG glia confirms that the existence of the Slit-Robo2 signaling pathway and the downstream molecule, srGAP3, in DRG neurons. In conclusion, we have shown that srGAP3 binds to the Slit1 receptor Robo2 to promote neurite outgrowth of DRG sensory neurons.

Growth cone protrusion and retraction of neurons is due to the dynamic assembly and disassembly of cytoskeletal proteins, such as tubulin and actin. RhoA, Cdc42, Rac1 GTPases provide the necessary integration sites for the complex regulation of growth cone motility, cell division, and actin dynamics in neuronal growth. Overexpression of dominant active forms of Rho initiate axon outgrowth and control of growth cone filopodial dynamics and promote axonal regeneration[39,40]. The Ras superfamily, has been shown to control the actin cytoskeleton through cycling between the active GTP-bound and inactive GDP-bound states[39,41]. Although srGAP1 downregulates Cdc42, the GAP domains of both srGAP2 and srGAP3 are specific for Rac1[31,35,36,42]. SrGAP3 is widely expressed in the developing brain and is implicated in the etiology of cognitive impairment[19,31]. Rac1 and its downstream target, WAVE1, play an essential role in the formation of lamellipodia in fibroblasts and regulate neuronal morphology[43-45]. Furthermore, the interplay of srGAP3 and WAVE1 is important for the development of dendritic spines in primary hippocampal neurons[46,47]. Here, we demonstrated that Rac1 is immediately activated in response to anti-srGAP3 antibody treatment in cultured DRG neurons. It can be speculated that the binding of srGAP3 to Rac1 directly stimulates the interaction with WAVE1 and inhibits the generation of Rac1-GTPase causing the subsequent filopodia formation and outgrowth of growth cones. Furthermore, we

observed high Rac1-GTP expression in cultured neurons treated with anti-srGAP3 or anti-Robo2 antibodies, while in contrast, low Rac1-GTP expression was detected by Western blot and immunostaining analyses following Slit1 protein treatment.

In summary, neutralization of endogenous srGAP3 with an anti-srGAP3 antibody decreases neurite length of cultured DRG neurons. SrGAP3 is a powerful DRG sensory neuron neurite outgrowth promoter factor, which inactivates Rac1.

Our data suggest that the different domains of srGAP3 function together to mediate the effects of the protein in the signaling cascade downstream of Robo2. Furthermore, Slit1 binding to the Robo2 receptor might initiate a signaling response leading to srGAP3 activation resulting in inactivation of Rac1 leading to actin organization in the growth cone.

Conflict of interest statement

We declare that we have no conflict of interest.

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