

Modulation of Rho GTPase Activity Alleviates Chondroitin Sulfate Proteoglycan-Dependent Inhibition of Neurite Extension

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The central nervous system (CNS) fails to regenerate after injury. A glial scar forms at the injury site, contributing to regenerative failure partly resulting from the chondroitin sulfate proteoglycans (CSPGs) in the glial scar. The family of Rho GTPases, which includes Cdc42, Rac1, and RhoA, is involved in growth cone dynamics. Although the response of neural cells to the inactivation of Rho when contacting myelin-related substrates, or CSPG, has been investigated, Rac1's and Cdc42's abilities to modulate CSPG-dependent inhibition have yet to be explored. In this study, a stripe assay was utilized to examine the effects of modulating all three Rho GTPases on neurite extension across inhibitory CSPG lanes. Alternating laminin (LN) and CSPG lanes were created and NG108-15 cells and E9 chick dorsal root ganglia (DRGs) were cultured on the lanes. By using the protein delivery agent Chariot, the neuronal response to exposure of constitutively active (CA) and dominant negative (DN) mutants of the Rho GTPases, along with the bacterial toxin C3, was determined by quantifying the percentage ratio of neurites crossing the CSPG lanes. CA-Cdc42, CA-Rac1, and C3 transferase significantly increased the number of neurites crossing into the CSPG lanes compared with the negative controls for both the NG108-15 cells and the E9 chick DRGs. We also show that these mutant proteins require the delivery vehicle, Chariot, to enter the neurons and affect neurite extension. Therefore, activation of Cdc42 and Rac, as well as inhibition of Rho, helps overcome the CSPG-dependent inhibition of neurite extension. © 2004 Wiley-Liss, Inc.

Key words: chondroitin sulfate proteoglycans; Rho GTPases; neurite inhibition; NG108-15 cells; dorsal root ganglia; regenerative failure

Physical injury to the central nervous system (CNS) often results in permanent functional loss, because astroglial scar at the site of injury results in a nonpermissive environment for regeneration. Astroglial scar contains as-

trocytes, oligodendrocytes, oligodendrocyte precursors, meningeal cells, and microglia that produce inhibitory molecules (Fawcett and Asher, 1999) that have been implicated in regenerative failure (Hoke and Silver, 1996; David and Lacroix, 2003). Inhibitory macromolecules include myelin-associated glycoproteins (MAG) (McKeracher et al., 1994; Mukhopadhyay et al., 1994), NOGO-A (Niederost et al., 2002), and oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002), which are myelin-related, and chondroitin sulfate proteoglycans (CSPGs), a family of nonmyelin molecules (for review see Spencer et al., 2003). CSPGs, consisting of a protein core and glycosaminoglycan (GAG) side chains (referred to as *chondroitin sulfate*) (Morgenstern et al., 2002), are classified as aggrecan, phosphacan, neurocan, brevican, NG2, and versican (Tang, 2003). CSPGs are mainly produced by astrocytes and are also expressed by oligodendrocyte precursors and meningeal cells (Fawcett and Asher, 1999).

There are two potential strategies, extrinsic and intrinsic, to regenerate nerves through the glial scar. Extrinsic approaches focus on removing inhibition, typically by using the enzyme chondroitinase ABC to cleave the GAG into its constituent disaccharides. This strategy helps to partially alleviate the CSPG-mediated inhibition in vitro and in vivo (McKeon et al., 1995; Zuo et al., 1998; Bradbury et al., 2002).

In this study, we investigate an intrinsic strategy of alleviating the inhibitory influence of CSPGs through modulation of Rho GTPases. The Rho GTPase family,

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particularly Cdc42, Rac1, and RhoA, are involved in the growth cone dynamics (Hall, 1998). Cdc42 induces filopodia growth, and Rac1 promotes lamellipodia formation (Nobes and Hall, 1995). RhoA responds to inhibitory cues by inducing growth cone collapse (Hall, 1998). Rho GTPases are active when GTP-bound and are inactive while GDP-bound. Mutant forms of the Rho GTPases that mimic either the GTP-bound, constitutively active (CA), or GDP-bound, dominant negative (DN), have been constructed (Coso et al., 1995; Kozma et al., 1995). We utilized CA- and DN-glutathione S-transferase (GST) fusion proteins of the Rho GTPases.

It has been reported previously that MAG, NOGO-A, and OMgp contribute to the CNS inhibition (for review see Filbin, 2003). Alleviation of myelin-related inhibition by inhibiting the activation of Rho and its downstream effector, ROCK, by using C3 transferase (C3) or Y27632, has been reported. Typically, these proteins are transduced into cells through trituration (Jin and Strittmatter, 1997). However, this disrupts cell function and reduces cell viability. Therefore, delivery reagents that transduce the Rho GTPases while preserving cell viability are needed. Therefore, TAT, a human immunodeficiency viral peptide, was fused to C3 to aid in protein uptake (Winton et al., 2002; Dubreuil et al., 2003; Monnier et al., 2003). Although these studies have focused mainly on Rho and ROCK inactivation on both myelin and CSPG surfaces, the effect of modulating Rac1 and Cdc42 on CSPG-dependent inhibition is still unclear.

By using a generic protein delivery agent, we investigated the effect of modulating Rac1 and Cdc42, as well as C3, on CSPG-mediated inhibition (specifically aggrecan). We used a modified *in vitro* Bonhoeffer stripe assay (Vielmetter et al., 1990), consisting of alternating lanes of laminin (LN) and CSPG (Snow et al., 2002; Chen et al., 2002; Monnier et al., 2003). CSPG lanes are nonpermissive for cellular attachment and neurite outgrowth (Schmalfeldt et al., 2000; Sango et al., 2003). LN was used to aid in neuronal attachment on the non-CSPG lanes (Condic and Lemons, 2002). NG108-15 cells and whole E9 chick dorsal root ganglia (DRGs) were added to the lanes and transduced with Rho GTPase mutant proteins. The percent ratio of neurites that grew along the LN lanes vs. the percent of neurites that crossed into the CSPG lanes was quantified as a measure of alleviation of CSPG-dependent inhibition.

MATERIALS AND METHODS

Surface Modification of Tissue Culture Dishes

Alternating CSPG and LN (Invitrogen, Carlsbad, CA) lanes were created on 60-mm-diameter tissue culture dishes by using a modified version of the Bonhoeffer method (Vielmetter et al., 1990). The CSPG (aggrecan), a gift from Dr. Arnold Caplan's laboratory (Department of Biology, Case Western Reserve University, Cleveland, OH), was purified from embryonic chick chondrocytes. The 60-mm dishes were initially coated with nitrocellulose (5 cm² nitrocellulose dissolved in 12 ml methanol) and air dried for 30 min. The Bonhoeffer silicone

matrices (Max Planck Institute, Tübingen, Germany) were placed over a uniform nitrocellulose region. CSPG was dissolved in distilled deionized water for a final concentration of 150 µg/ml, and 25 µg/ml of bovine albumin serum (BSA)-fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Milwaukee, WI) was added for visualization. This mixture was injected into the matrix chamber, aspirated into the channels, and allowed to bind to the nitrocellulose for 10 min. The CSPG solution was then aspirated out of the channels, and the procedure was repeated four additional times. Next, 2% BSA (Sigma) was aspirated through the channels and allowed to incubate for 10 min to block any unbound sites. This cycle was performed three times. Phosphate-buffered saline (PBS) was aspirated into the channels three times in rapid succession to remove any unbound protein. The silicone matrix was removed, and LN (40 µg/ml) was added over the modified region for 30 min. To block the unbound nitrocellulose, 2% BSA was added to the entire dish for 10 min. After removal of the BSA, the dishes were rinsed with Dulbecco's Modified Eagle's Media (DMEM; Invitrogen, Carlsbad, CA), then stored at 37°C with 2 ml of DMEM to be used within a few hours.

NG108-15 Cells Neurite Outgrowth Assays

NG108-15 cells (ATCC, Manassas, VA) were removed from T75 culture flasks by trypsinization and then resuspended in Neurobasal-A media (Invitrogen, Carlsbad, CA) supplemented with N-2, L-glutamine, and 1% penicillin-streptomycin. The DMEM was removed from the modified tissue culture dishes, and 2 ml of Neurobasal-A media along with 2×10^4 NG108-15 cells were added over the lane region. The cultures were incubated for 4 hr in 37°C and 5% CO₂. By 4 hr, NG108-15 cells typically formed concise lanes, adhering only to LN lanes and not to CSPG lanes. The Rho GTPases, L61 Cdc42(CA)-GST, N17 Cdc42(DN)-GST, L61 Rac1(CA)-GST, N17 Rac1(DN)-GST, and L63 RhoA(CA)-GST, as well as C3 transferase (Cytoskeleton, Denver, CO), were prepared for protein transduction by complexing them with Chariot (described below). For the GST control, the GST fusion protein was complexed to the Chariot. To form the protein transduction complexes, 8 µl of 1 mg/ml protein in 200 µl PBS and 20 µl Chariot (Active Motif, Carlsbad, CA), which had been dissolved previously in sterile deionized distilled water, was further diluted in 200 µl of deionized distilled water, and the two solutions were mixed and incubated at room temperature for 30 min. For the C3, the molar equivalent (2 µg/ml) of the other mutant proteins was transduced. Four hours after plating of the cells, 400 µl of the Neurobasal-A medium was removed from the culture, and the protein/Chariot complex was added to the dish. For the media control, sterile deionized distilled water and PBS were added to the cultures without protein or Chariot. Five hours after plating, 1 ml of Neurobasal-A media was added, for a final culture media volume of 3 ml. The cultures were placed in humidified incubators at 37°C and 5% CO₂ and were imaged under light microscopy after 48 hr as described below. The cultures were then fixed by using 4% paraformaldehyde and stored for further analysis.

A dose-response study was performed with the following final concentrations of CA-Cdc42, 1 µg/ml, 2 µg/ml, 3 µg/ml,

4 $\mu\text{g}/\text{ml}$, 6 $\mu\text{g}/\text{ml}$, and 8 $\mu\text{g}/\text{ml}$. The culturing, incubation, and protein transduction were performed as described above.

To evaluate whether the Chariot reagent aids proteins in entering the cells, protein transductions with CA-Rac1 and C3 were performed without the addition of Chariot. The same procedures were performed as described above; however, the same solution was made without the Chariot reagent.

DRG Neurite Outgrowth Assays

Sixty-millimeter culture dishes with alternating lanes of LN and CSPG were prepared as described above. Whole E9 White Leghorn chicks were dissected, and three or four whole DRGs were cultured on each of the modified tissue culture dishes. Neurobasal medium (500 μl) supplemented with B-27 supplement and L-glutamine was added to the culture dishes. The protein/Chariot complexes were prepared with 8 μl of 1 mg/ml of protein and 20 μl of Chariot and added to the DRGs for 1 hr at 37°C as described above for NG108-15 cells. At the end of the hour, another 500 μl of Neurobasal medium were added. The cultures were imaged after 48 hr as described below and then fixed with 4% paraformaldehyde.

Quantification of Neurite Crossing

The NG108-15 cells and DRGs were imaged and analyzed by using ImagePro software (Media Cybernetics, Carlsbad, CA) through a Magnafire CCD camera (Optronics, Goleta, CA) attached to an inverted Nikon T300 microscope. For the cultures with NG108-15 cells, the neurites were counted and categorized into two groups: 1) neurites that extended along LN lanes and 2) processes that crossed into the CSPG lanes. Neurites were counted only in the regions where cells attached and formed distinct lanes. A percent ratio of neurites crossing the CSPG lane was calculated based on the equation:

$$\frac{\text{No. of neurites crossing into CSPG lane}}{\text{No. of neurites growing on the LN lanes}} \cdot 100$$

For the cultures with whole DRGs, the results were quantified in a blinded state by two, independent, unbiased people. Each DRG explant in the cultures was scored from 0 to 4, similar to the scoring system used by Walter et al. (1987) to quantify the amount of neurite crossing from retinal explants. The score of 0 is given to explants that exhibit a preference for either LN or CSPG substrate, and 4 is given to explants with neurites that show no preference for either of the two substrates. The scores 1–3 were given for intermediate neurite crossing. At least three DRGs per condition were analyzed by each of the two blinded people, and their scores were averaged.

Statistical Analysis

The Student's *t*-test was performed with Minitab software. Conditions were considered to be statistically different for *P* values < .05.

RESULTS

The percent ratio of neurite crossing into the CSPG lanes was measured for the two negative controls (no protein transduction and GST transduction) as well as

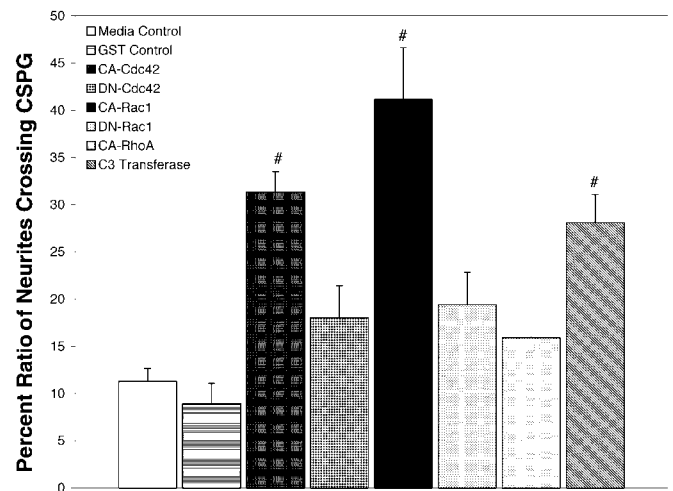


Fig. 1. Percent ratio of neurites crossing for the controls, Rho GTPase mutant proteins, and C3 transferase. After treatment with CA-Cdc42, CA-Rac1, or C3, the percent ratio of neurites crossing CSPG increased. The data represent the mean \pm SEM for three experiments. #Statistically different compared with media control (*P* < .05).

Chariot complexed to C3 and the Cdc42, Rac1, and RhoA mutants (Fig. 1). The two negative controls, containing only media or GST protein, complexed with Chariot, displayed few neurites crossing the CSPG lanes (11.3% and 8.9%, respectively). The mutant protein, CA-Rac1, had the highest percentage of crossing at 41.1%, followed by CA-Cdc42 and C3, which had crossing percentages of 31.3% and 28.1%, respectively, which were all statistically significant compared with the negative controls.

In Figure 2, phase micrographs illustrate the effect of the mutant proteins, C3, and the negative controls. The neurites extended on the LN lane or along the CSPG/LN border under the control conditions (Fig. 2A,B). CA-Cdc42, CA-Rac1, and C3 had the greatest amount of neurites crossing the CSPG lane (Fig. 2C–E). The DN mutants and CA-RhoA had little effect on neurite extension (Fig. 2F–H).

A dose-response experiment was conducted on CA-Cdc42 with six different protein concentrations; 1 $\mu\text{g}/\text{ml}$, 2 $\mu\text{g}/\text{ml}$, 3 $\mu\text{g}/\text{ml}$, 4 $\mu\text{g}/\text{ml}$, 6 $\mu\text{g}/\text{ml}$, and 8 $\mu\text{g}/\text{ml}$ (Fig. 3). Only the lower four concentrations are plotted on this graph; the higher doses (6 $\mu\text{g}/\text{ml}$, and 8 $\mu\text{g}/\text{ml}$) were toxic and did not permit cell attachment. As shown in Figure 3, the percent ratio of crossing was 14.7%, 14.4%, and 12.7% for 1 $\mu\text{g}/\text{ml}$, 2 $\mu\text{g}/\text{ml}$, and 3 $\mu\text{g}/\text{ml}$ CA-Cdc42 sample groups, respectively. The concentration that elicited the highest percent ratio of crossing was 4 $\mu\text{g}/\text{ml}$ CA-Cdc42 (31.3%). Only the 4 $\mu\text{g}/\text{ml}$ CA-Cdc42 sample group was statistically different from the media control.

Figure 4 graphically portrays the effect of using the Chariot transduction system. CA-Rac1 was used to investigate whether Chariot was necessary to aid protein uptake

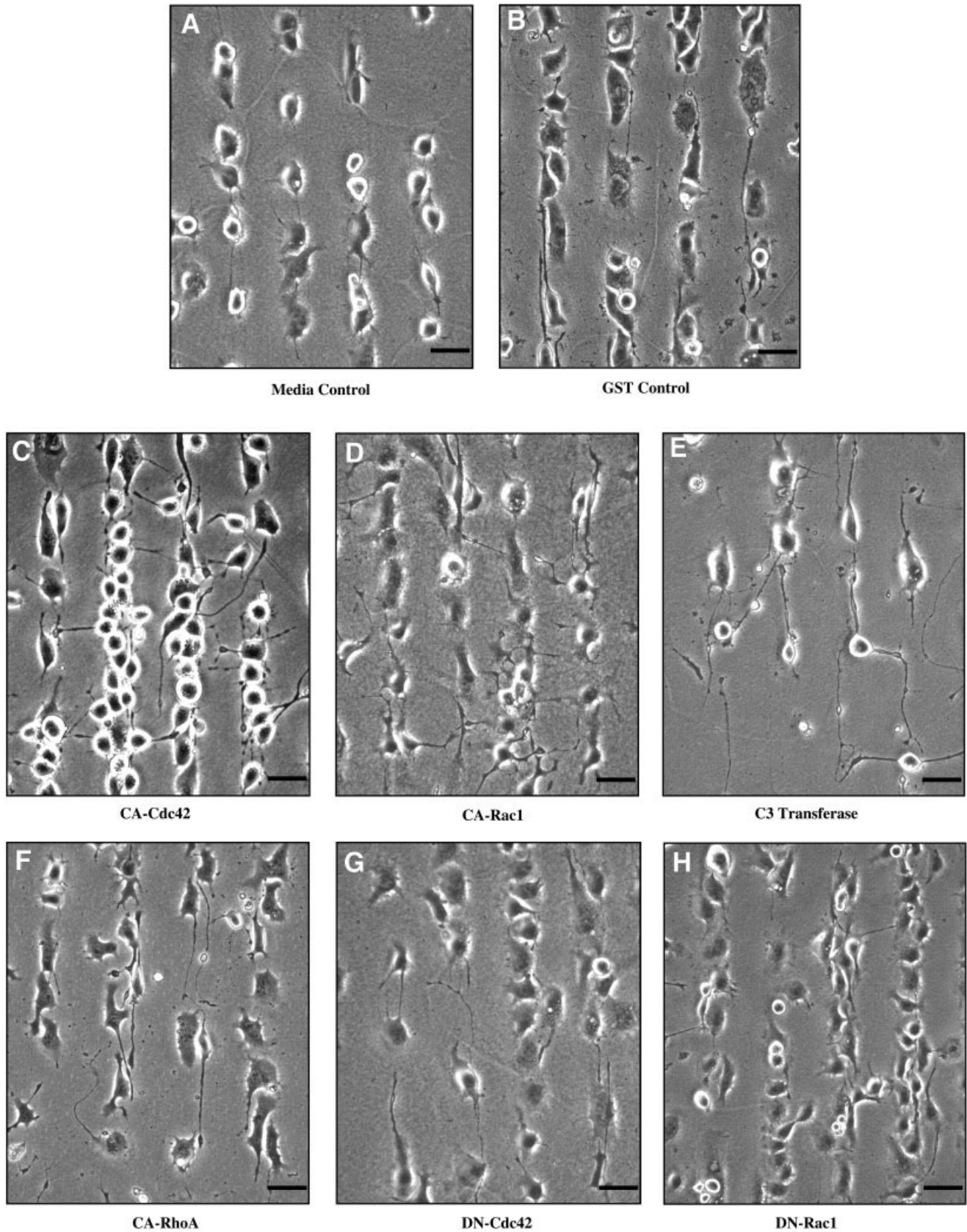


Fig. 2. NG108-15 cells transduced with mutant Rho GTPases and C3. **A:** Media control. **B:** GST control. **C:** CA-Cdc42. **D:** CA-Rac1. **E:** C3 transferase. **F:** CA-RhoA. **G:** DN-Cdc42. **H:** DN-Rac1. The neurons attached on the laminin lanes, and the neurites grew predominantly along the laminin/CSPG border. CA-Cdc42, CA-Rac1, and

C3 (C-E) induced neurites to cross the CSPG lanes. The neurites in the two controls, DN-Cdc42, DN-Rac1, and CA-RhoA (A,B,F-H) sample groups grew on the laminin lanes with very little or no crossing of the CSPG lanes. Scale bars = 50 μm.

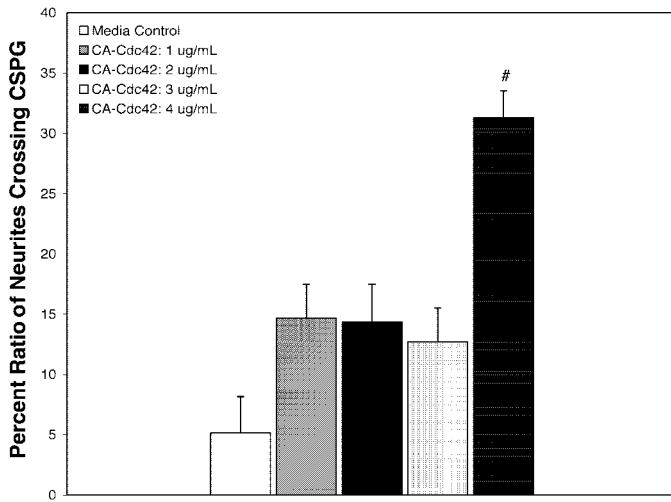


Fig. 3. CA-Cdc42 dose-response curve for NG108-15 cells. For CA-Cdc42, 4 $\mu\text{g}/\text{mL}$ induced the highest percent ratio of neurites crossing the CSPG lanes. The data represent the mean \pm SEM for three experiments. [#]Statistically different compared with media control ($P < .05$).

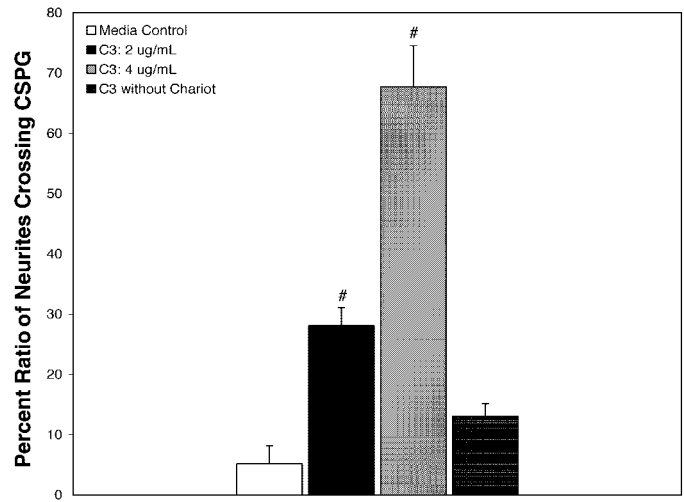


Fig. 5. C3 transferase with and without Chariot. When the dosage of C3 was doubled, the percentage ratio of crossing increased. Without the Chariot, the percentage ratio of neurites crossing is similar to that of the media control. The data represent the mean \pm SEM for three or four experiments. [#]Statistically different compared with media control ($P < .05$).

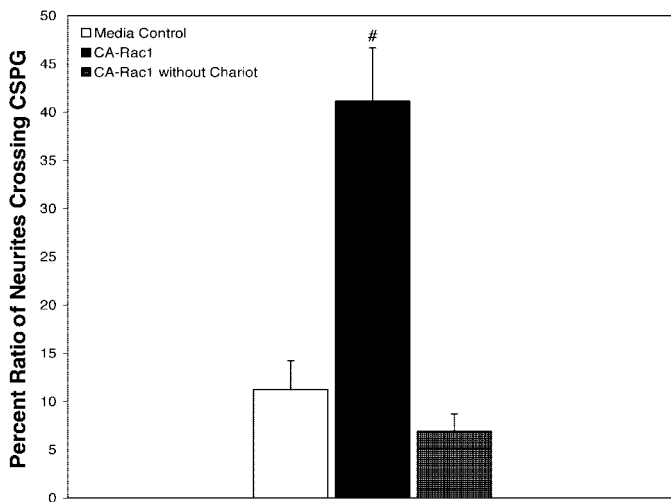


Fig. 4. Percent ratio of neurites crossing after CA-Rac1 treatment with and without Chariot. CA-Rac1 with Chariot elicited the highest percentage of crossing. CA-Rac1 without the Chariot had a percent ratio similar to that of the media control. The data represent the mean \pm SEM for three experiments. [#]Statistically different compared with media control ($P < .05$).

by the cells. Figure 4 demonstrates that there was a significantly greater number of neurites that crossed the CSPG lane when the mutant protein was complexed to the delivery reagent compared with the negative control. The average percent ratio of crossed neurites through the inhibitory region in the absence of Chariot was lower than the media control with an average of 6.9%.

C3 was administered as the molar equivalent of the other mutant proteins (Fig. 1). However, in a separate

experiment, the molar amount of C3 was doubled (4 $\mu\text{g}/\text{mL}$) so that we could observe the effect on neurite extension through the CSPG lanes. When comparing the different C3 sample groups with the media control, 2 $\mu\text{g}/\text{mL}$ and 4 $\mu\text{g}/\text{mL}$ of C3 were statistically greater (Fig. 5). C3 was added to the cells without Chariot as well. The percent ratio of neurites crossing was similar to that of the media control (13%).

To determine whether the effect observed in the NG108-15 cells would be similar in primary neurons, CA-Cdc42, CA-Rac1, DN-Rac1, and C3 proteins were transduced into whole E9 chick DRGs. Figure 6 depicts the results for whole DRGs neurites extending along the LN and CSPG regions. A modified version of the preference scoring system developed by Walter et al. (1987) was used for analysis. The results demonstrate that, in the media control, the neurites had a score of 1.2 with a strong preference for the laminin regions. CA-Cdc42 and CA-Rac1 had scores of 3 and 2.9, respectively, with greater number of neurites crossing over into the CSPG regions. The neurite crossing for C3 scored a 2, and DN-Rac1 scored a 1.5. In Figure 7, phase micrographs of the DRG cultures at 48 hr after plating are shown. The lower portion of each image distinguishes between LN lanes (gray) and CSPG lanes (black). The media control shows the majority of the neurites in tight bundles growing along the LN regions, with a few neurites crossing the CSPG lanes (Fig. 7A). Bundles of neurites extending along the LN lanes can also be seen in the sample groups when CA-Cdc42, C3, or CA-Rac1 was transduced into the E9 DRGs; however, there are also increased amounts of neurites crossing the CSPG inhibitory lanes (Fig. 7B-D). The sample group that was transduced with DN-Rac1 was

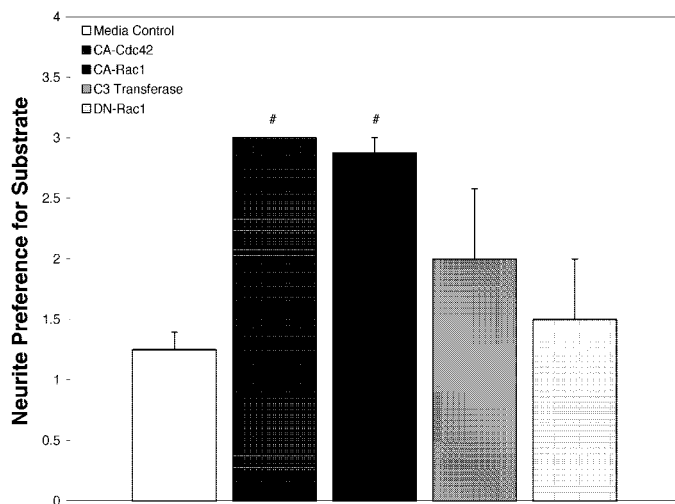


Fig. 6. DRG neurite preference for a substrate. A score of 0 means that neurites preferred to extend on only one substrate, either LN substrate or CSPG. A score of 4 means that the neurites do not show a preference for a specific substrate. A score between 1 and 3 indicates that there is a preference for one substrate; however, the neurites will extend along the other regions. After treatment with CA-Cdc42 and CA-Rac1, the neurites show less of a preference for laminin and extended along the CSPG lanes. The data represent the mean \pm SEM for three experiments. [#]Statistically different compared with the media control ($P < .05$).

similar to the media control, with neurites growing along the LN lanes and very few crossing into the CSPG lane (Fig. 7E). Therefore, CA-Cdc42, C3, and CA-Rac1 increased the number of neurites extending into the CSPG lane in both NG108-15 cells and primary DRGs.

DISCUSSION

Nerve regeneration in the CNS is currently limited. Our hypothesis is that CSPGs in glial scars are partially responsible for CNS regenerative failure by specifically sending inhibitory signals to the neurons, blocking regeneration. Understanding the signaling pathways involved in blocking neurite outgrowth through the scar has been difficult. However, knowledge of the signals involved in this region would illuminate the strategies that promote regeneration through the scar. We hypothesized that, regardless of the precise nature of the signals, they must converge on molecules that regulate the actin cytoskeleton, which are necessary for neurite outgrowth. The Rho GTPases represent a point of convergence for many extracellular signals that regulate actin polymerization. Our strategy was either to block the inhibitory signals or to mimic positive signals to stimulate neurite extension through inhibitory interfaces. Therefore, we modulated the activity of the Rho GTPases (Rac1, Rho, and Cdc42), which play important roles in regulating the actin cytoskeleton (Hall and Nobes, 2000; Luo, 2000). The Rho GTPases have also been implicated in axon guidance and in neurite outgrowth (Kuhn et al., 2000). We demonstrate

that this strategy is effective in overcoming CSPG-dependent inhibition of neurite outgrowth in an in vitro model system. We demonstrate that activation of Rac1 and Cdc42, as well as inhibition of Rho, promotes neurite outgrowth through CSPG regions. We speculate that the CSPG-mediated inhibition was alleviated either by modulating intracellular signaling to frustrate CSPG's inhibitory role or by altering the cytoskeletal mechanics so that repulsive forces were overcome.

Specifically, the effect of the mutant Rho GTPases proteins on the NG108-15 neurites' ability to cross into CSPG regions was quantified (Fig. 1). All the mutant proteins were transduced into the NG108-15 cells by using the Chariot system. The proteins that promoted neurite crossing, C3, CA-Cdc42, and CA-Rac1, had a significantly higher percent ratio of neurites crossing into the CSPG lanes compared with the negative controls. CA-Cdc42 and CA-Rac1 were also statistically greater than DN-Cdc42, DN-Rac1, and CA-Rho, indicating that these two proteins in their active state promote neurite extension into CSPG regions. Through the inactivation of RhoA signal transduction pathway, C3 had a significantly higher number of neurites crossing than DN Cdc42 and CA-RhoA when 2 μ g/ml of C3 was transduced. When a higher concentration of C3 (4 μ g/ml) was transduced, the condition was statistically greater than DN-Cdc42, DN-Rac1, and CA-RhoA. Therefore, these results demonstrate that transducing CA-Rac1 and CA-Cdc42 complexed to Chariot, as well as C3, can increase the number of neurites extending from the LN lanes into the CSPG lanes.

To determine whether combinations of mutant proteins produced synergistic effects, CA-Cdc42 and CA-Rac1 were cotransduced into the same lane-modified culture dish such that the total mutant protein concentration was the same as with the single protein alone (4 μ g/ml) combining 2 μ g/ml of CA-Cdc42 with 2 μ g/ml of CA-Rac1. The percent ratio of crossing for this condition was about 13% (data not shown). This percent ratio is similar to that observed when 2 μ g/ml were transduced alone (Fig. 3). When higher concentrations of CA-Cdc42 and CA-Rac1 were cotransduced into the NG108-15 cells (total concentrations of 6 μ g/ml and 8 μ g/ml), the cells did not survive, although they remained attached to the surface, probably as a result of toxicity associated with the protein transduction. Therefore, there was no observed synergistic effect of adding CA-Cdc42 and CA-Rac1.

To determine whether there was a dose response to the mutant protein, six different concentrations of CA-Cdc42 were added (Fig. 3). CA-Cdc42 was chosen for the dose-response curve because of its positive effect on neurites crossing the CSPG lanes (Fig. 1). At the higher concentrations (6 μ g/ml and 8 μ g/ml), cell death occurred probably as a result of cytotoxicity from either the protein itself or the Chariot reagent. At higher concentration, basic proteins in the cell such as the Rho GTPases, may potentially trigger a signal to begin apoptosis, leading

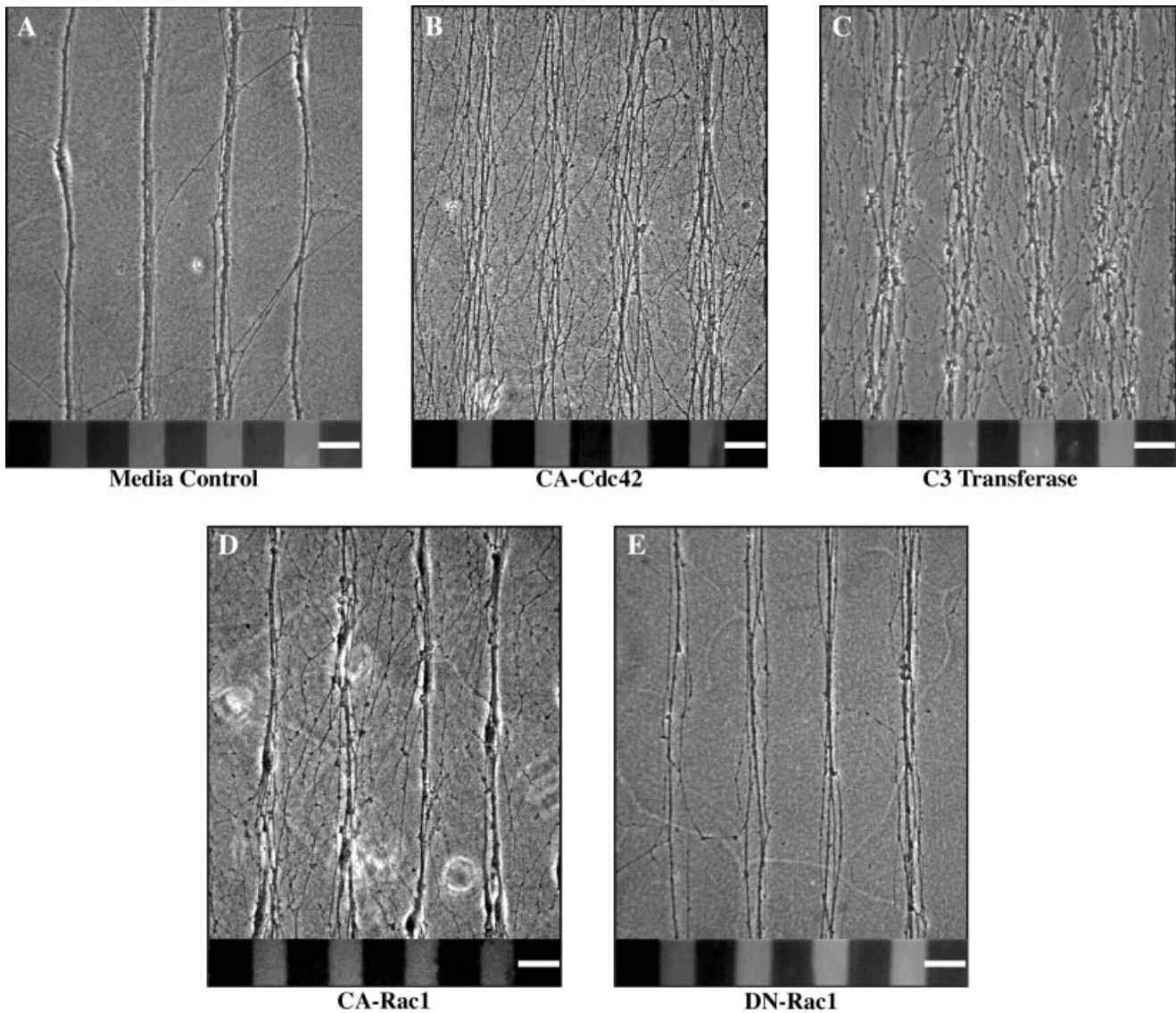


Fig. 7. DRGs transduced with mutant Rho GTPases and C3. **A:** Media control. **B:** CA-Cdc42. **C:** C3 transferase. **D:** CA-Rac1. **E:** DN-Rac1. Three to five experiments were conducted per condition. Note that under each neurite image is an image that shows LN lanes in gray and CSPG lanes in black. Similarly to the NG10815 cells, CA-Cdc42, CA-Rac1, and C3 (B–D) induced neurites to cross the CSPG lanes. The neurites in the media control and the DN-Rac1 (A,E) sample groups grew on the laminin lanes with very little crossing. Scale bars = 50 μ m.

to cell death. The three lower concentrations of protein did not evoke a substantial increase compared with the negative controls in the percent average of neurites crossing the lanes. The narrow range of protein concentration that leads to cellular responses shows that there is a concentration optimal for observing the desired response. Therefore, the percent ratio of neurites crossing the CSPG lane was more of a rectangular response rather than a peak response. The percentages of neurites crossing in wells transduced with the lower three concentrations of CA-Cdc42 were statistically lower compared with dishes with

4 μ g/ml of CA-Cdc42, which produced an average neurite crossing that was twofold higher. Therefore, the concentration of 4 μ g/ml of CA-Cdc42 was optimal for eliciting a crossing response from the neurons under our culture conditions. This validates the mutant protein concentrations used for the experiment comparing the effects of all of the Rho GTPase mutants.

Three methods commonly utilized to express a specific protein in cells, trituration (Jin and Strittmatter, 1997), microinjection (Paterson et al., 1990), and scrape loading (Lehmann et al., 1999), are aggressive and can

disrupt normal cell function. Therefore, it is necessary to engineer a method of transducing proteins efficiently without risking cell viability and administering the proteins *in vivo*. In some studies, the TAT peptide was used to aid in protein transduction (Winton et al., 2002; Dubreuil et al., 2003; Monnier et al., 2003). In this study, the Chariot reagent was used to transduce the proteins across the cellular membrane. Thus far, none of the previous studies has complexed protein with a generic delivery vehicle to aid in the protein transduction. To investigate the contribution of Chariot, both CA-Rac1 and C3 (two of the three conditions with the highest percentage crossing through the CSPG lanes; Fig. 4) were added to the neurons without any Chariot (Figs. 4, 5). Figure 4 shows that CA-Rac1 complexed with Chariot had a six-fold higher percent ratio of neurites crossing the lane compared with when the protein was added directly to the cultures, which makes it significantly higher. When the Chariot was excluded during the addition of C3, the percent ratio of crossing decreased by more than twofold (Fig. 5). These experiments confirm that the Rho GTPase mutant proteins and C3 have a limited ability to enter the cells on their own and require the aid of a delivery vehicle to enter the cells and influence neurite extension on CSPG.

C3 was the only protein transduced into the neurons that is not a member of the Rho GTPase family. The other mutants are CA or DN forms of the Rho GTPases. However, C3 is used to inhibit the activation of the RhoA. The molecular weight of C3 (24 kDa) is approximately half that of the other mutant proteins (50–52 kDa); therefore, 2 $\mu\text{g}/\text{ml}$ were used in a previous experiment as a molar equivalent (Fig. 1). To observe the effect of doubling the molar concentration, 4 $\mu\text{g}/\text{ml}$ of C3 was complexed to Chariot. The percent ratio increased by more than twofold (Fig. 5). The percent ratio of neurite crossing after transducing 4 $\mu\text{g}/\text{ml}$ of C3 was significantly greater than 2 $\mu\text{g}/\text{ml}$ of C3, and both of these conditions were statistically greater than C3 without the Chariot reagent. This demonstrates that a higher concentration of C3 can promote greater numbers of neurites crossing the inhibitory CSPG lanes *in vitro*.

Most studies investigating Rho GTPases have focused on RhoA, rather than examining Rac1- and Cdc42-dependent regulation of regeneration. The addition of C3 or Y27632 blocks myelin and myelin/CSPG inhibition *in vitro* (Dergham et al., 2002) as well as CSPG inhibition (Borisoff et al., 2003). Another study demonstrated that, on NOGO and myelin substrates, the percentage of neurite outgrowth increased after the administration of C3 (Fournier et al., 2003). In *in vitro* stripe assay experiments, C3 was fused to TAT, and these studies demonstrated that inactivating the Rho pathway overcame CSPG inhibition (Monnier et al., 2003) as well as myelin (Winton et al., 2002) and NOGO (Dubreuil et al., 2003). The use of TAT as a delivery vehicle requires a tedious process to construct the mutant protein. Chariot is convenient because it is a generic delivery reagent that can be complexed

to any protein and works with high efficiency. There has been conflicting evidence on the benefits of adding C3 to the corticospinal tract to achieve long-distance regeneration (Dergham et al., 2002; Fournier et al., 2003). One of the theories for C3 failing to generate axon growth over long-distance gaps is that the Rho inhibitor is not successfully transduced into the injured cells at the site (Fournier et al., 2003).

E9 whole DRGs were cultured to determine whether the results obtained with the NG108-15 cells could be replicated in primary neurons. CA-Cdc42 and CA-Rac1 were transduced into the cells and induced a significantly greater amount of crossing, similar that of the NG108-15 cells. This demonstrates that, although neurites preferred the LN substrate, after CA-Cdc42 or CA-Rac1 was transduced, the neurites were able to extend on CSPG lanes. Although C3 was not significantly different compared with the media control, transducing the protein did evoke a neurite crossing into the CSPG region. DN-Rac1 was used as the negative control in this experiment and did not encourage neurites to cross into the inhibitory region, as shown in Figures 6 and 7. Obtaining similar results from two different cell types allows the conclusion that CA-Cdc42, CA-Rac1, and C3 stimulate growth cone extension into the CSPG lane and overcome its inhibitory effects. It would be interesting to evaluate the utility of CA-Cdc42 and CA-Rac1 transduction *in vivo* to overcome regenerative failure in the CNS.

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