Rac1 and Cdc42 but Not RhoA or Rho Kinase Activities Are Required for Neurite Outgrowth Induced by the Netrin-1 Receptor DCC (Deleted in Colorectal Cancer) in NIE-115 Neuroblastoma Cells*

Xiaodong Li†‡§, Etienne Saint-Cyr-Proulx‡, Klaus Aktories¶ and Nathalie Lamarche-Vane‡‖

From the †Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec H3A 2B2, Canada and the ‡Institut für Pharmakologie und Toxikologie der Universität Freiburg, Hermann-Herder-Strasse 5, Freiburg D-79104, Germany

Netrins are chemotropic guidance cues that attract or repel growing axons during development. DCC (deleted in colorectal cancer), a transmembrane protein that is a receptor for netrin-1, is implicated in mediating both responses. However, the mechanism by which this is achieved remains unclear. Here we report that Rho GTPases are required for embryonic spinal commissural axon outgrowth induced by netrin-1. Using NIE-115 neuroblasts, we found that both Rac1 and Cdc42 activities are required for DCC-induced neurite outgrowth. In contrast, down-regulation of RhoA and its effector Rho kinase stimulates the ability of DCC to induce neurite outgrowth. In Swiss 3T3 fibroblasts, DCC was found to trigger actin reorganization through activation of Rac1 but not Cdc42 or RhoA. We detected that stimulation of DCC receptors with netrin-1 resulted in a 4-fold increase in Rac1 activation. These results implicate the small GTPases Rac1, Cdc42, and RhoA as essential components that participate in signaling the response of axons to netrin-1 during neural development.

Netrins are a small family of secreted proteins that guide growing axons during neural development (1, 2). The first netrin cloned, UNC-6, was identified using a genetic screen for mutations affecting axon guidance in Caenorhabditis elegans (3). Netrins were first identified in vertebrates on the basis of their ability to promote commissural axon outgrowth from explants of embryonic spinal cord (4, 5). Netrin family members have now been identified in multiple vertebrate and invertebrate species and shown to have a highly conserved function as axon guidance cues (6). Netrins are chemotropic guidance cues that attract or repel growing axons during development (1, 2). The first netrin cloned, UNC-6, was identified using a genetic screen for mutations affecting axon guidance in Caenorhabditis elegans (3). Netrins were first identified in vertebrates on the basis of their ability to promote commissural axon outgrowth from explants of embryonic spinal cord (4, 5). Netrin family members have now been identified in multiple vertebrate and invertebrate species and shown to have a highly conserved function as axon guidance cues (6). Netrins are bifunctional molecules that can attract or repel growing axons during development, depending on the extracellular environment and the netrin isoform expressed.UNC-5 represents the first netrin receptor identified in C. elegans, and it is implicated in transducing the netrin signal to the neuronal growth cone (12).

The intracellular mechanisms mediating the response of an axon to netrin-1 are currently unclear. Previous studies indicate that extracellular guidance cues induce the neuronal growth cone to advance, retract, or turn by regulating the actin cytoskeleton within the growth cone (13). The Rho family of small GTPases, in particular, RhoA, Rac1, and Cdc42, are well established regulators of actin reorganization in non-neuronal cells (14), and there is now compelling evidence demonstrating roles for RhoA, Rac1, and Cdc42 as signaling elements within the neuronal growth cone (15, 16). Here, we report that members of the Rho family of GTPases are required for commissural axon outgrowth produced by netrin-1 from explants of embryonic rat spinal cord. Both Rac1 and Cdc42 are required for neurite outgrowth promoted by DCC in NIE-115 neuroblasts, and in contrast, inhibition of RhoA and Rho kinase increases the ability of DCC to induce neurite outgrowth. In Swiss 3T3 cells, DCC was found to trigger actin reorganization through activation of Rac1 in a netrin-1-dependent manner. In fibroblasts, DCC did not activate Cdc42 or RhoA.

**EXPERIMENTAL PROCEDURES**

**Explant Assay—**Embryonic day 13 rat dorsal spinal cord and floor plate explants were dissected and cultured in three-dimensional collagen gels as described (5). Recombinant chick netrin-1 protein was produced and purified as described (5). Toxin B was purified as previously described (17). Both netrin-1 protein and toxin B were added to the culture medium at the beginning of the culture period. The explants were cultured for 14 h, then fixed with 4% paraformaldehyde, and photographed with an Optronics MagnaFire camera and a Carl Zeiss Axiovert microscope using a 20× objective lens and phase contrast optics. The length of axon fascicles growing out of the explants were quantified using Northern Eclipse Software (Empix Imaging). The total length of fascicle growth was then calculated for each explant.

**DNA Constructs—**Standard DNA protocols were used as described (18). pRK5-DCC-C (3394–4690 bp) was generated by digestion of pBS-DCC with EcoRI and BglII followed by ligation of a EcoRI-BglII fragment into pRK5 digested with EcoRI and BamHI. To generate pRK5 encoding full-length DCC, a fragment (3061 bp) from the start codon to the EcoRI site of pBS-DCC was amplified by PCR and subcloned into pRK5-DCC-C digested with EcoRI, pDCC-E encoding the N terminus of DCC (1122 amino acids) comprising the extracellular and transmembrane domains tagged with green fluorescent protein (GFP)† at its C-terminal end. pDCC-E was generated by subcloning the extracellular and transmembrane domains of DCC into pRK5-DCC-C digested with EcoRI.

† The abbreviations used are: GFP, green fluorescent protein; RT, reverse transcriptase; WASP, Wiskott-Aldrich syndrome protein; GST, glutathione S-transferase.
Role of Rho GTPases in DCC-induced Signaling Pathway

**Fig. 1.** Toxin B inhibits netrin-1-dependent outgrowth of commissural axons. A illustrates the quantification of axon outgrowth from explants of E13 rat dorsal spinal cord shown in B–G. Bar C, control; Bar N, netrin-1 (200 ng/ml). B, a negative control explant cultured in the absence of netrin-1. C, axon outgrowth in the presence of 200 ng/ml netrin-1 protein. Netrin-1-dependent (200 ng/ml) axon outgrowth was reduced by the addition of toxin B to the cultures. D, 1.0 ng/ml; E, 0.1 ng/ml; F, 0.01 ng/ml; G, 0.001 ng/ml. The arrowhead in E indicates a rounded cell body present at the edge of the explant. The scale bar in G corresponds to 100 μm, and the scale is the same in B–G. The error bars represent the S.E. and n = 4 in each case.

Terminus was kindly provided by Dr. Tim Kennedy (McGill University). DNA was purified using a Qiagen kit. For microinjection studies, purified plasmids were filtered through a 0.2-μm cellulose acetate membrane (Corning) before microinjection into cells.

Reverse Transcriptase (RT)-PCR—Total RNA was purified using Trizol (Invitrogen) and poly(A)+ RNA was isolated using the Oligotex mRNA purification kit (Qiagen). First strand cDNA was synthesized using superscript reverse transcriptase (Invitrogen). PCR was used to amplify cDNA using the following primers: UNC5 h1, GGA ATT CCC TCC CTC GAT CCC AAT GTG T and TCC CCG GGC GGC ACG GAA CGA AAG TAG T, 909 bp; UNC5 h2, GCT CTA GAG TCG CGG CAG CAG GTG GAG GAA and GGA ATT CAG GGG GCG GCT TTT AGG GTC GTT, 771 bp; and DCC, CCG CTC GAG TGG TCA CCG CAG CTC TCA and GCC TGG ATC TTC TGT TGG CTT GCT, 938 bp. The primers were annealed at 60 °C, and 35 cycles of amplification were carried out. The size of the predicted amplification product is indicated.

Cell Culture and Microinjection—Mouse fibroblast Swiss 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and antibiotics and maintained in an atmosphere of 10% CO2. Confluent serum-starved Swiss 3T3 cells were prepared as described (19). Briefly, the cells were plated in 5% serum at a density of 6 × 104 onto acid-washed coverslips. 7–10 days later, the cells became quiescent and were subjected to serum starvation for 16 h in Dulbecco’s modified Eagle’s medium containing 2 g/liter NaHCO3. The eukaryotic expression vector pRK5 encoding full-length DCC or pRK5-DCC-C was kindly provided by Dr. Tim Kennedy (McGill University). pDCC-E or pEGFP were microinjected at 0.1 mg/ml into the nucleus of COS-7 cells. 24 h after transfection, the cells were serum-starved overnight and treated with netrin-1 (500 ng/ml) for different periods of time.

Immunofluorescence Microscopy—At the indicated times, microinjected Swiss 3T3 cells or transfected N1E-115 cells were rinsed with phosphate-buffered saline and fixed for 10 min in freshly prepared 4% (w/v) paraformaldehyde. All steps were carried out at room temperature, and coverslips were rinsed in phosphate-buffered saline between each of the steps. The cells were permeabilized in 0.2% Triton X-100 for 5 min, and free aldehyde groups were reduced with 0.5 mg/ml sodium borohydride for 10 min. The cells were double labeled following the procedure previously described (21). Briefly, the cells were incubated with the primary monoclonal antibodies anti-DCC (Pharmingen, G97-449), anti-Myc (a generous gift from Dr. Nicole Beauchemin, McGill University), or anti-GFP (Molecular Probe) diluted in phosphate-buffered saline for 60 min. Then coverslips were transferred to a second antibody mixture composed of fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Sigma) and tetramethylrhodamine isothiocyanate-conjugated phallolidin (Sigma) for 60 min. N1E-115 cells were fixed in 3% glutaraldehyde for 1 h, rinsed in PBS, and stained positively for neurofilament M using a polyclonal anti-neurofilament M (Chemicon). Coverslips were mounted by inverting them onto 8 μl of Mowiol (Calbiochem) mounting containing p-phenylene diamine as an anti-bleach reagent. After 2 h at room temperature, the coverslips were examined on a Zeiss Axiosvert 135 microscope using Zeiss oil immersion 63× objective lens. Fluorescence images were recorded using a digital camera (DVC) and analyzed with Northern Eclipse software (Empix Imaging Inc.).

**Fig. 1 continued**

**A** Total length of axons (0.1 mm) per explant, μm

| toxin B, ng/ml | 0 | 0.001 | 0.01 | 0.1 | 1.0 |
|---------------|---|------|------|-----|-----|
| C             | 2000 | 1000 | 500  | 100 | 0   |
| N             | 2000 | 1000 | 500  | 100 | 0   |
| T             | 2000 | 1000 | 500  | 100 | 0   |

**B** Bar C, control; Bar N, netrin-1 (200 ng/ml).

**C** Axon outgrowth in the presence of 200 ng/ml netrin-1 protein. Netrin-1-dependent (200 ng/ml) axon outgrowth was reduced by the addition of toxin B to the cultures. D, 1.0 ng/ml; E, 0.1 ng/ml; F, 0.01 ng/ml; G, 0.001 ng/ml. The arrowhead in E indicates a rounded cell body present at the edge of the explant. The scale bar in G corresponds to 100 μm, and the scale is the same in B–G. The error bars represent the S.E. and n = 4 in each case.

**D** The size of the predicted amplification product is indicated.

**E** Cell Culture and Microinjection—Mouse fibroblast Swiss 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and antibiotics and maintained in an atmosphere of 10% CO2. Confluent serum-starved Swiss 3T3 cells were prepared as described (19). Briefly, the cells were plated in 5% serum at a density of 6 × 104 onto acid-washed coverslips. 7–10 days later, the cells became quiescent and were subjected to serum starvation for 16 h in Dulbecco’s modified Eagle’s medium containing 2 g/liter NaHCO3. The eukaryotic expression vector pRK5 encoding full-length DCC or pRK5-DCC-C was microinjected alone or with pRK5 encoding Myc-tagged Cdc42N17, RacN17, or pEFmyc-C3 transferase into COS-7 cells. The cells were permeabilized in 0.2% Triton X-100 for 5 min, and free aldehyde groups were reduced with 0.5 mg/ml sodium borohydride for 10 min. The cells were double labeled following the procedure previously described (21). Briefly, the cells were incubated with the primary monoclonal antibodies anti-DCC (Pharmingen, G97-449), anti-Myc (a generous gift from Dr. Nicole Beauchemin, McGill University), or anti-GFP (Molecular Probe) diluted in phosphate-buffered saline for 60 min. Then coverslips were transferred to a second antibody mixture composed of fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Sigma) and tetramethylrhodamine isothiocyanate-conjugated phallolidin (Sigma) for 60 min. N1E-115 cells were fixed in 3% glutaraldehyde for 1 h, rinsed in PBS, and stained positively for neurofilament M using a polyclonal anti-neurofilament M (Chemicon). Coverslips were mounted by inverting them onto 8 μl of Mowiol (Calbiochem) mounting containing p-phenylene diamine as an anti-bleach reagent. After 2 h at room temperature, the coverslips were examined on a Zeiss Axiosvert 135 microscope using Zeiss oil immersion 63× objective lens. Fluorescence images were recorded using a digital camera (DVC) and analyzed with Northern Eclipse software (Empix Imaging Inc.).

**Fig. 1 continued**

**F** Purification of GST-PAK and GST-WASP—GST-PAK (amino acids 56–272) and GST-WASP (amino acids 201–321) were used to isolate GTP-bound Rac1 and Cdc42, respectively. Escherichia coli transformed with GST-PAK and GST-WASP constructs were grown at 37 °C to an absorbance of 0.5. Expression of the fusion proteins was induced by isopropyl-β-D-thiogalactopyranoside (1 mM) for 3 h at 37 °C. The cells were washed once in STE buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) prior to sonication in buffer A (20 mM Hepes, pH 7.5, 120 mM NaCl, 2 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). The lysates were cleared by centrifugation, and Nonidet P-40 was added to a final concentration of 0.5%. The proteins were stored at −80 °C until use. Protein concentration was determined by comparison with different...
Fig. 2. Expression of netrin-1 and netrin-1 receptors in N1E-115 cells and Swiss 3T3 fibroblasts. A, the expression of DCC and netrin-1 proteins was detected by Western blot analyses using monoclonal anti-DCC (upper panel) and polyclonal PN3 anti-netrin-1 (lower panel) antibodies, respectively. Upper panel, lane 1, 50 μg of protein lysate from DCC-expressing Cos-7 cells; lane 2, 100 μg of Swiss 3T3 protein cell lysate; lane 3, 100 μg of serum-starved N1E-115 protein cell lysate. Lower panel, lane 1, 100 ng purified netrin-1; lane 2, 100 μg of Swiss 3T3 protein cell lysate; lane 3, 100 μg of serum-starved N1E-115 protein cell lysate. B, RT-PCR amplification of cDNA from poly(A)+ RNA isolated from newborn mouse brain (lane 1), Swiss 3T3 cells (lane 2), or serum-starved N1E-115 cells (lane 3). Lane 4 is a negative control containing no cDNA. DNA markers (lanes M) show the 100-bp DNA ladder (New England Biolabs).

Fig. 3. DCC-induced neurite outgrowth requires Rac1 and Cdc42 activity in N1E-115 neuroblastoma cells. N1E-115 neuroblastoma cells were transfected either with empty vector pRK5 (G) or pRK5-DCC alone (A and B) or with pRK5myc-RacN17 (C and D) or pRK5myc-Cdc42N17 (E and F). F-actin (B, D, F, and G) was visualized with fluorescently tagged phalloidin, and DCC (A, C, and E) was revealed by costaining with an anti-DCC antibody and by indirect immunofluorescence. The expression of Myc-tagged RacN17 and Cdc42N17 was detected using anti-Myc antibodies and by indirect immunofluorescence. Scale bars, 10 μm. The scale in A and B is different from that in C–G.

RESULTS

Rho GTPases Are Required for Commissural Axon Outgrowth Evoked by Netrin-1—To determine whether Rho GTPases are involved in mediating the axon outgrowth promoting activity of netrin-1, we examined the effect of adding the Rho GTPase inhibitor toxin B to explants of E13 rat dorsal spinal cord cultured in a three-dimensional collagen gel in the presence of netrin-1. Explants of dorsal spinal cord cultured in the presence of recombinant netrin-1 (200 ng/ml) produced maximal commissural axon outgrowth from these explants as previously reported (4) (Fig. 1, A and C). The addition of increasing concentrations of toxin B from 0.001 to 1 ng/ml in the presence of maximal concentrations of netrin-1 resulted in increasing inhibition of commissural axon outgrowth from explants (Fig. 1, A and D–G). In the presence of toxin B, cells at the edge of the explants were clearly rounded (Fig. 1E, arrowhead), a characteristic effect of toxin B on other cell types (23). In addition, although a small amount of axon outgrowth occurred at higher concentrations of toxin B, the axons were much less fasciculated than normal (Fig. 1, compare C with D and E). These findings indicate that netrin-1-mediated commissural axon outgrowth requires the activity of one or more Rho GTPases.
DCC-induced Neurite Outgrowth Requires Rac1 and Cdc42 but Not RhoA or Rho Kinase Activities in N1E-115 Neuroblastoma Cells—Mouse N1E-115 neuroblastoma cells exhibit neurite outgrowth in response to serum deprivation (24). Using immunoblotting analyses, N1E-115 cells were found to constitutively express netrin-1. However, these cells did not express DCC (Fig. 2A). RT-PCR analysis revealed the expression of mRNAs encoding the netrin-1 receptors UNC5 h1 and UNC5 h2 but not DCC (Fig. 2B). In the presence of 5% serum, the cells are round and extend lamellipodia and multiple filopodia (Fig. 3G). When DCC is expressed in N1E-115 cells in the presence of 5% serum, 62% of transfected cells exhibited neurite outgrowth (Fig. 4). The majority of DCC-expressing cells contained one long neurite (~30 μm) per cell with thin filopodia along the neurite (Fig. 3, A and B). DCC protein was consistently enriched at the extending tip of the neurite as shown in Fig. 3A (arrow). The presence of antibodies blocking the function of DCC or netrin-1 inhibited the ability of DCC to induce neurite outgrowth in N1E-115 cells (Fig. 4). In addition, truncated DCC proteins lacking the majority of the extracellular domain (DCC-C) or the cytoplasmic domain (DCC-E) of DCC were unable to produce neurite outgrowth (Fig. 4). These results strongly suggest that netrin-1 binding to DCC is necessary to mediate intracellular signaling events leading to neurite outgrowth in N1E-115 cells.

C3 transferase has been shown to inactivate RhoA by ADP-ribosylation at residue Asn41 (23). When C3 transferase is expressed in N1E-115 cells, 50% of transfected cells showed neurite outgrowth as previously reported (53) (Fig. 4). When DCC is expressed in the presence of C3 transferase, the number of transfected cells with neurite outgrowth increased to 80% (Fig. 4). Similarly, when DCC-expressing cells are incubated with the Y-27632 compound that inhibits the Rho effector Rho kinase, neurite outgrowth is stimulated in more than 80% of transfected cells (Fig. 4). These results suggest that inhibition of RhoA and its effector Rho kinase known to mediate the effects of RhoA on neurite retraction in N1E-115 cells (51) increases the ability of DCC to stimulate neurite extension in N1E-115 cells.

Expression plasmids encoding dominant negative RacN17 or Cdc42N17 were transfected together with pRK5-DCC into N1E-115 cells. As shown in Fig. 3 (D and F), both dominant negative Rac1 and Cdc42 significantly inhibited neurite outgrowth induced by DCC. In DCC-expressing cells, the dominant negative Rac1 and Cdc42 mutants reduced neurite extension by 55 and 45%, respectively (Fig. 4). Cells expressing both RacN17 and DCC were rounded and flattened and exhibited long filopodia but not lamellipodia. These findings suggest that although Rac1 has been inhibited, Cdc42 remained activated in these cells (Fig. 3D). Cells expressing both Cdc42N17 and DCC exhibited lamellipodia and short microspikes at the plasma membrane, suggesting that Rac1 remained activated in these cells (Fig. 3F). Therefore, both Rac1 and Cdc42 activities are required for neurite outgrowth induced by DCC in N1E-115 cells.

The Netrin-1 Receptor DCC Activates Rac1 but Not Cdc42 or RhoA in Swiss 3T3 Fibroblasts—To further dissect the mechanisms used by netrin-1 to signal through Rho GTPases, we reconstituted the phenomenon in Swiss 3T3 fibroblasts by transiently expressing DCC and using the organization of the actin cytoskeleton as a functional read-out. Following serum starvation, Swiss 3T3 cells lose most of the actin based structures usually found in a fibroblast: lamellipodia, filopodia, and stress fibers. However, the cells do remain attached to the supporting extracellular matrix (Fig. 5A). Microinjection of constitutively active Cdc42L61, RacL61, and RhoL63 proteins into quiescent, serum-starved Swiss 3T3 cells has been shown to rapidly induce the formation of three distinct actin based structures: filopodia, lamellipodia, and stress fibers, respectively (19, 25–27). In addition, in some cell types, such as fibroblasts and epithelial cells, activation of Cdc42 leads to rapid activation of Rac1, which in turn leads to activation of RhoA (26). Netrin-1 and DCC proteins were undetectable by Western blot analyses of Swiss 3T3 cell lysates (Fig. 2A). RT-
PCR analyses detected the expression of mRNAs encoding UNC5 h2 but not UNC5 h1 or DCC. As shown in Fig. 5B, the addition of recombinant netrin-1 protein does not affect the reorganization of polymerized actin in uninjected cells. Microinjection of the eukaryotic expression vector, pRK5, encoding full-length rat DCC into quiescent, serum-starved Swiss 3T3 cells led to the expression of DCC (Fig. 5C, E, and G) were visualized as described in the legend to Fig. 3. Approximately 100 cells were microinjected per coverslip, and 5 h after injection, 90% of the injected cells showed expression of DCC. Scale bars, 10 μm. The arrows indicate localization of DCC at the plasma membrane (E) and membrane ruffles (F).

FIG. 5. Cytoskeletal changes in Swiss 3T3 cells induced by netrin-1 receptor DCC. Serum-starved Swiss 3T3 cells microinjected with pRK5-DCC (C–H) were fixed after either no addition (A, C, and D) or addition of 500 ng/ml recombinant netrin-1 for 10 min (E and F) or 30 min (B, G, and H). F-Actin (A, B, D, F, and H) and DCC (C, E, and G) were visualized as in Fig. 3. Approximately 100 cells were microinjected per coverslip, and 5 h after injection, 90% of the injected cells showed expression of DCC. Scale bars, 10 μm. The arrows indicate localization of DCC at the plasma membrane (E) and membrane ruffles (F).

The Netrin-1 Receptor DCC Promotes Rac1 GTP Loading—Pull-down assays were carried out in which Rac1 and Cdc42 GTP loading was assessed by specific binding of the active GTPases to the Cdc42/Rac interactive binding domain (29) of DCC expressed in Swiss 3T3 fibroblasts. As shown in Fig. 6B, the addition of 100 ng/ml recombinant netrin-1 for 30 min, suggesting that netrin-1 binding to DCC is essential to activate Rac GTPase signaling pathways (Fig. 6B). Similarly, the expression of a truncated DCC protein lacking the cytoplasmic domain and coupled to green fluorescent protein did not lead to actin reorganization after the addition of netrin-1 (Fig. 6D). We conclude that DCC is essential to activate the cascade of Rac GTPases in Swiss 3T3 cells in a ligand-dependent manner.

To determine whether DCC activates the cascade of Rac GTPases through Cdc42 or Rac1 in Swiss 3T3 fibroblasts, we microinjected quiescent, serum-starved Swiss 3T3 cells with pRK5-DCC together with eukaryotic vectors encoding either Myc-tagged dominant negative RacN17 or Cdc42N17 or C3 transferase. As shown in Fig. 7, the expression of dominant negative Cdc42N17 did not inhibit actin reorganization induced by netrin-1 in DCC-expressing cells (Fig. 7, compare D with B), whereas dominant negative RacN17 inhibited the formation of both lamellipodia and stress fibers (Fig. 7F). C3 transferase blocked the formation of stress fibers but not the formation of polymerized actin at the leading edge of the plasma membrane (Fig. 7H). Hence, DCC activates Rac1-induced signaling pathways but not Cdc42-dependent signals in Swiss 3T3 fibroblasts. These findings indicate that in these cells, activation of RhoA by DCC is a consequence of cross-talk between Rac1 and RhoA.

FIG. 6. Netrin-1 binding to DCC is essential to mediate actin reorganization in Swiss 3T3 fibroblasts. Serum-starved Swiss 3T3 cells microinjected with pRK5-DCC-C (A and B), with pDCC-E (C and D), or with pEGFP (E and F) were fixed after addition of 500 ng/ml recombinant netrin-1 for 30 min. F-Actin (B, D, and F) and DCC-C (A) were visualized as in Fig. 3. DCC-E fused to GFP at its C terminus (C) and GFP (E) were visualized using anti-GFP antibodies and indirect fluorescence. Approximately 100 cells were microinjected per coverslip, and 5 h after injection, 90% of the injected cells showed expression of DCC. Scale bars, 10 μm.
p65PAK or WASP (Wiskott-Aldrich syndrome protein) fused to glutathione S-transferase (GST-PAK or GST-WASP), respectively, DCC and Myc-tagged Rac1 or Cdc42 were coexpressed in COS-7 cells for 24 h, and the cells were serum-starved overnight followed by the addition of netrin-1 to the medium. The lysates were prepared, and the amount of Rac1 or Cdc42 precipitated with GST-PAK or GST-WASP, respectively, was determined by Western blot analysis. Netrin-1 stimulated a 4-fold increase in the level of activated Rac1 (Fig. 8, A and B), whereas no increase in GTP-Cdc42 was observed after stimulation with netrin-1 (Fig. 8, C and D). The cells expressing Rac1 in the absence of DCC showed no increase in Rac1-GTP after 5 min of stimulation with netrin-1, suggesting that DCC is required for Rac1 activation. These data are consistent with the microinjection studies in Swiss 3T3 cells, suggesting that netrin-1 receptor DCC activates Rac1 but not Cdc42 in fibroblasts.

**DISCUSSION**

Netrin-1 and its receptor, DCC, are widely expressed in embryonic and adult tissues (4, 30–32, 52). Their function in many cell types is poorly understood, but in the embryonic central nervous system they act as attractive and repulsive cues that guide the migration of developing axons (8). Here we demonstrate that toxin B inhibits commissural axon outgrowth evoked by netrin-1, thereby implicating Rho GTPases in mediating the effect of netrin-1 on these axons. Both Rac1 and Cdc42 were found to be necessary for DCC-induced neurite outgrowth in N1E-115 neuroblastoma cells. When RhoA and Rho kinase were inhibited, respectively, by C3 transferase or Y-27632 in N1E-115 cells, 80% of DCC-expressing cells exhibit neurite outgrowth, suggesting that down-regulation of RhoA and Rho kinase is required for DCC to induce neurite outgrowth in N1E-115 cells. In fibroblasts, the expression of DCC triggered actin reorganization in a netrin-1-dependent manner through the activation of Rac1 but not RhoA or Cdc42. Netrin-1 stimulation of DCC resulted in a 4-fold increase of Rac1 activation without affecting the level of activated Cdc42. Interestingly, these results suggest that a neuronal-specific guanine nucleotide exchange factor required for DCC to activate Cdc42 may be absent in fibroblasts. Alternatively, a specific coreceptor for netrin-1 that is required for DCC to activate Cdc42 may not be expressed in fibroblasts. Altogether, this study provides compelling evidence for a key role of regulated activities of Rac1, Cdc42, and RhoA in the cytosolic signaling mechanisms induced by DCC when it binds to netrin-1. Consistent with our findings, it has been reported that some of the defects caused by an activated form of the C. elegans DCC homolog, UNC-40, could be partly suppressed by mutations in Ced-10, a member of the Rac family in C. elegans (33).

In addition to the formation of lamellipodia in DCC-expressing fibroblasts, DCC also induced the formation of stress fibers as a result of cross-talk between Rac1 and RhoA. A current model suggests that attractive guidance cues activate Rac1 or Cdc42 and inhibit RhoA to promote directed axonal outgrowth, whereas repulsive cues inhibit Rac1 or Cdc42 and stimulate RhoA to induce retraction (15, 16). In support of this model, Wahl et al. (34) showed that Ephrin-A5 activates Rho and inhibits Rac in cultured retinal ganglion cells. Here we propose that when a growth cone is attracted by netrin-1, DCC may activate Rac1 while inhibiting RhoA in neuronal cells. It may be the case that the activation of RhoA by Rac1 in fibroblasts reported here is restricted to non-neuronal cells.

The implication that second messengers, Ca\(^{2+}\) and cAMP, modulate the response to netrin-1 has emerged from *in vitro* studies of growth cone turning using *Xenopus* spinal neurons (35–38). Using the same assay, coactivation of phosphatidylinositol 3-kinase and phospholipase C\(\gamma\) pathways were shown to be required for the turning response of the growth cone (39). Phosphatidylinositol 3-kinase mediates activation of Rac1 downstream of many tyrosine kinase receptors (40). However, it has not yet been determined whether phosphatidylinositol 3-kinase links DCC to activation of Rac1 upon binding to netrin-1. Protein kinase A phosphorylation of RhoA and the intracellular level of cAMP negatively regulate the activity of RhoA in different cell types (41), and the inhibition of RhoA and of its effector, Rho kinase, is required for cAMP-induced outgrowth of dendrites in B16 cells (42). The effects shown here...
mediated by DCC may be a consequence of a coordinated activation of Rac1 leading to actin polymerization at the advancing edge of the growth cone and inactivation of RhoA through the maintenance of the intracellular levels of cAMP in neurons.

The cytoplasmic domain of DCC did not interact physically with Rac1 (data not shown), suggesting an indirect link between DCC and Rac1. A candidate protein that may link DCC to activation of Rac1 is the UNC-73 ortholog Trio, a guanine nucleotide exchange factor with activity toward both Rac1 and RhoA (43), found to play a major role in axonal development and pathfinding (44–48). The cytoplasmic tail of DCC contains several putative SH3-binding motifs, PXXP (49), that may interact with the two SH3 domains of Trio or to an SH3-containing adapter molecule.

Before its discovery as an axon guidance receptor in the nervous system, DCC was identified as a tumor suppressor gene in colorectal cancer and appears to play a role in the development of the nervous system, DCC was identified as a tumor suppressor gene in colorectal cancer and appears to play a role in the development of the nervous system, DCC was identified as a tumor suppressor gene in colorectal cancer and appears to play a role in the development of the nervous system.

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Fig. 8. Netrin-1 receptor DCC promotes Rac GTP loading. COS-7 cells were transfected with 5 μg of pRK5 or pRK5-DCC together with 1.5 μg of pRK5myc-Rac1 (A and B) or pRK5myc-Cdc42 (C and D) and treated with netrin-1 (500 ng/ml) for the indicated times. The cells were lysed, and the equal amount of protein was incubated with GST-PAR (amino acids 52–272) and GST-WASP (amino acids 201–321) (C and D) protein coupled to glutathione-Sepharose beads. Upper panel, GTP-bound Rac1 (A) or GTP-bound Cdc42 (C) was detected by Western blotting using anti-Myc antibodies. Lower panel, total cell lysates probed for Rac1 (A) or Cdc42 (C) demonstrated equal amounts of GTPase. B and D illustrate a time course of the fold change of Cdc42 and Rac1 activation following the addition of netrin-1 to cells expressing DCC. The fold activation of GTPase was determined by densitometry, and the values correspond to the averages of at least three independent experiments.

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Role of Rho GTPases in DCC-induced Signaling Pathway

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