The Adaptor Protein Nck-1 Couples the Netrin-1 Receptor DCC (Deleted in Colorectal Cancer) to the Activation of the Small GTPase Rac1 through an Atypical Mechanism*

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Netrins are a family of secreted proteins that guide the migration of cells and axonal growth cones during development. DCC (deleted in colorectal cancer) is a receptor for netrin-1 implicated in mediating these responses. Here, we show that DCC interacts constitutively with the SH3/SIH2 adaptor Nck in commissural neurons. This interaction is direct and requires the SH3 but not SH2 domains of Nck-1. Moreover, both DCC and Nck-1 associate with the actin cytoskeleton, and this association is mediated by DCC. A dominant negative Nck-1 inhibits the ability of DCC to induce neurite outgrowth in N1E-115 cells and to activate Rac1 in fibroblasts in response to netrin-1. These studies provide evidence for an important role of mammalian Nck-1 in a novel signaling pathway from an extracellular guidance cue to changes in the actin-based cytoskeleton responsible for axonal guidance.

In the developing nervous system, newborn neurons extend their axons to their targets in response to attractive and repulsive cues. The neuronal growth cone, located at the tip of the growing axon, is a highly motile structure that can be viewed as a sophisticated signal transduction device capable of recognizing extracellular guidance cues and translating them into directed neurite outgrowth (1, 2). Over the past years, a combination of cellular and genetic studies has led to the identification of several proteins playing a critical role in guiding axons along their pathways, including members of the ephrin, semaphorin, netrin, slit, reelin, and nerve growth factor families of proteins (3). Several classes of transmembrane proteins have also been identified and characterized as their respective binding receptors (4). For instance, two classes of receptors, DCC and the UNC-5 proteins, have been described for the netrins. Netrins are bifunctional molecules attracting and repelling different classes of axons. The DCC family of receptors mediates growth cone attraction by netrins (5–8), whereas the UNC-5 proteins are required for the repulsive effect of the netrins (9–11). However, the pathway is more complicated since UNC-5-mediated repulsion requires the function of DCC proteins in some cases (12–14).

We recently determined that the small GTPases Rac1, Cdc42, and RhoA play a key role in the cytosolic signaling events induced by the netrin-1 receptor DCC (15, 16). Several putative Src homology 3 (SH3)1 binding motifs, PXXP (17), are found in the cytoplasmic tail of DCC, suggesting a possible interaction with an SH3-containing adaptor molecule to mediate netrin-1 signaling to Rho GTPases. A role for the fly protein Dock in axon guidance has been well characterized during Drosophila eye and nervous system development (18–21). Nck, the mammalian homolog of Dock, is a ubiquitously expressed protein composed of a single SH2 and three SH3 domains and is represented by two genes (22, 23).

In this paper, we provide biochemical and functional evidence for the involvement of Nck in mediating the netrin-1 receptor DCC signaling. We demonstrate that DCC associates with Nck in embryonic spinal commissural neurons. The interaction of DCC and Nck is independent of netrin-1 and involves the direct binding of DCC to two SH3 domains of Nck-1. Furthermore, DCC associates with the actin cytoskeleton and is responsible for the relocalization of Nck-1 to the Triton-insoluble fraction. In N1E-115 neuroblastoma cells, a dominant negative Nck-1 inhibits the ability of DCC to induce neurite outgrowth. In fibroblasts, dominant negative Nck-1 blocks the activation of Rac1 by DCC in the presence of netrin-1. Thus, we propose that Nck-1 is constitutively associated with DCC through two SH3 domains. In the presence of netrin-1, Nck-1 bound to DCC is now able to interact with downstream effectors via the SH2 domain to mediate changes in the actin-based cytoskeleton through activation of Rac1.

EXPERIMENTAL PROCEDURES

Plasmids—pRK5 encoding full-length DCC (pRK5-DCC) was as described previously (15). pGEX4T-2 encoding the carboxyl terminus of DCC (425 amino acids) comprising the cytoplasmic transmembrane domain and 61 amino acids of the extracellular domain was produced by PCR amplification of pRK5-DCC-C (15) followed by ligation of the PCR product into pGEX4T-2 digested with EcoR1 and Xhol. pCDNAmyc encoding Nck-1 or the various mutant proteins and GST fusion proteins of Nck-1 and mutant proteins were as described previously (24). GST fusion proteins encoding the carboxyl-terminal SH3 of Grb2 (amino

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The abbreviations used are: SH3, Src homology 3; SH2, Src homology 2; GST, glutathione S-transferase; PBS, phosphate-buffered saline; CdGAP, Cdc42 GTPase-activating protein.
DCC associates with Nck in commissural neurons from E13 rat spinal cord. Lysates of newborn rat brains (A) or commissural neurons incubated with or without netrin-1 for 10 min at 160 ng/ml (B) or COS-7 cells transfected with pcDNA-myc-Nck-1 alone or together with pRK5-DCC (C) were submitted to immunoprecipitation (IP) using monoclonal anti-Nck antibodies (IP αNck) or with mouse immunoglobulin G coupled to protein G-Sepharose beads (IP IgG). Total cell lysates (TCL) and immunoprecipitated proteins (IP) were submitted to SDS-PAGE, and DCC and Nck were detected by Western blotting (WB) using monoclonal anti-DCC (WB aDCC) and polyclonal anti-Nck (WB αNck) antibodies, respectively. D, the level of DCC and Nck proteins co-immunoprecipitated from commissural cell lysates in B was quantified by densitometry and is expressed as arbitrary units.

Acids 159–217, the SH3 domains of Src, intersectin, and the proline-rich domain of Cdc42 were described elsewhere (25, 26). 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.

Preparation of Total Cell Lysates from Newborn Rat Brains and Commisural Neurons—Whole brains were extracted from newborn rats and incubated in 10 ml (per brain) of a buffer containing 20 mM Heps, pH 7.2, 1% Triton X-100, 10% bovine serum albumin, 100 mM NaCl, 20 mM sodium fluoride, 1 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Brain tissues were homogenized using a Dounce homogenizer and incubated for 30 min on ice. Lysates were cleared by a 10-min centrifugation at 14,000 rpm, and supernatants were passed several times through 25-gauge needles. The supernatants were then subjected to immunoprecipitation and immunoblotting. Commisural neurons were obtained by dissecting E13 rat embryo dorsal spinal cords as described previously (16). Briefly, 5 × 107 cells were plated onto 13-mm coverslips coated with poly-D-lysine and laminin-1 or on laminin-1-coated 60 mm dishes and maintained for 48 h after dissection in neurobasal medium (Invitrogen) containing 10% inactivated fetal bovine serum. After treatment or not for 10 min at 37 °C with 160 ng/ml netrin-1, commissural neurons plated on coverslips were subjected to immunofluorescence, whereas 60-mm plated cells were lysed and subjected to immunoprecipitation and immunoblotting.

Cell Culture and Microinjection—Mouse fibroblast Swiss 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics and maintained in an atmosphere of 10% CO2. Confluent serum-starved Swiss 3T3 cells were prepared as described (27). Briefly, cells were plated in 10% serum at a density of 6 × 105 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 was microinjected alone or with GST, GST-Nck-(SH33/SH33/SH33) or -Nck-(SH31/SH32/SH33) into the nucleus of ~100 cells over a period of 20 min in CO2-independent medium (Invitrogen) using Eppendorf microinjection system 5246. During microinjection, cells were maintained at 37 °C within a humidified atmosphere. Cells were returned to the incubator for a further 5 h before the addition of purified netrin-1 at 500 ng/ml for 10 min.

Mammalian Cell Transfection—N1E-115 neuroblastoma cells and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics at 10% CO2. N1E-115 cells were plated onto coverslips previously coated with laminin (20 μg/ml; VWR Canlab) for 24 h at 37 °C, washed twice with water, and left to air-dry. Transfection was carried out with LipofectAMINE transfection reagent (Invitrogen) according to the manufacturer’s protocol and as previously described (15). 0.4 μg of pRK5 or pRK5-DCC or either 0.4 μg of pcDNAmyc-Nck-1, -Nck (R308K)1–377, -Nck (W38R, W143R, W229R)1–377, or -Nck-(SH33/SH33/SH33)1–251 were used. COS-7 cells were transfected using the DEAE-dextran method as described previously (28). The amount of plasmids used per 100-mm dish was as follows: pRK5, 5 μg; pRK5-DCC, 2.5 μg; pcDNAmyc-Nck-1, 2.5 μg. Twenty-four hours after transfection, cells were either serum-starved overnight or not and treated with netrin-1 at 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 PBS and fixed for 10 min in freshly prepared 4% paraformaldehyde. All steps were carried out at room temperature, and coverslips were rinsed in PBS between each of the steps. 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. Cells were double-labeled after the procedure previously described (15). Briefly, cells were incubated with monoclonal anti-DCC antibodies (Pharmingen, G97–449), polyclonal anti-Nck antibodies (Upstate Biotechnology, 06–288), or anti-vinculin (Sigma) diluted in PBS for 60 min. Then, coverslips were
transferred to a secondary antibody mixture composed of fluorescein isothiocyanate-conjugated goat anti-mouse (Sigma) or goat anti-rabbit (Sigma) and tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma) for 60 min. For vinculin staining, cells were incubated with a tertiary fluorescein isothiocyanate-conjugated donkey anti-goat antibodies for an additional 30 min. Coverslips were mounted by inverting them onto 8 μl of Mowiol containing p-phenylenediamine as an anti-bleach reagent. After that room temperature, the coverslips were examined on a Zeiss Axiovert 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.).

In N1E-115 cells, a neurite was defined as a process that measured at least the length of a cell body.

Dissected E13 rat dorsal commissural neurons treated or not for 10 min at 37 °C with 160 ng/ml netrin-1 were rapidly rinsed with PBS, fixed in 4% paraformaldehyde for 10 min, and then permeabilized with 0.25% Triton X-100 in PBS for 5 min at room temperature. Blocking was performed for 1 h in 5% heat-inactivated normal goat serum. Coverslips were incubated overnight at 4 °C with monoclonal anti-DCC antibody (2 μg/ml) and polyclonal anti-Nck (2 μg/ml, Upstate Biotechnology). Cells were then gently washed with PBS and incubated with tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse and fluorescein isothiocyanate-conjugated goat anti-rabbit (Sigma) antibodies for 1 h at room temperature. Coverslips were mounted as described above. Coverslips were analyzed using a Zeiss LSM410 inverted laser-scanning confocal microscope, and images were captured with a 63× objective lens. Acquired images were analyzed using Zeiss LSM software.

**Immunoprecipitation—**Newborn rat brains, dissociated commissural neurons treated or not with 160 ng/ml netrin-1, or COS-7 cells transfected with pcDNAmyc-Nck-1 alone or together with pHK5-DCC were lysed in buffer containing 20 mM Hepes, pH 7.2, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 1 mg/ml bovine serum albumin, 20 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. 1 μg of protein lysates were precleared with 20 μl of protein G-Sepharose beads (Amersham Biosciences) at 4 °C overnight. The supernatants were incubated with normal mouse IgGs (Pierce) or with monoclonal anti-Nck (Upstate Biotechnology, 05-160) antibody at 4 °C for 2 h and with 10 μl of protein G-Sepharose beads for an additional hour. Beads were washed three times in lysis buffer and boiled in SDS sample buffer. Proteins were separated on 7.5% SDS-PAGE and revealed by immunoblotting using anti-DCC and polyclonal anti-Nck antibodies (29) and the ECL reagent detection kit (PerkinElmer Life Sciences). The level of immunoprecipitated DCC and Nck proteins obtained from commissural cell lysates was determined by densitometry (Bio-Rad imaging densitometer GS-700).

**Purification of GST Fusion Proteins—**Escherichia coli transformed with various GST fusion proteins were grown at 37 °C for 1 h. Expression of the fusion proteins was induced by isopropyl-β-D-thiogalactopyranoside (1 mM) for 3 h at 37 °C, except for GST-p65PAK, which was induced at room temperature. Cells were sonicated in buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The lysates were cleared by centrifugation, and Triton X-100 was added to a final concentration of 1% followed by incubation with glutathione-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. Beads were washed 3 times with buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride). GST fusion proteins were

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**Fig. 2.** DCC and Nck colocalize in the cell bodies, along the axons, and in growth cones of commissural neurons. DCC and Nck were visualized in commissural neurons treated with (B) or without netrin-1 (A) by co-staining with monoclonal anti-DCC and polyclonal anti-Nck antibodies, respectively, and by indirect immunofluorescence using a Zeiss 410 confocal microscope (upper panels). Lower panels represent enlargement of growth cones. Picture merge was obtained using Zeiss LSM software. Scale bar, 10 μm.
eluted in Buffer D (50 mM Tris, pH 8, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) containing 0.005 mM glutathione (Sigma).

**Dot Blot Assay**—The interaction of the cytoplasmic tail of DCC expressed as a GST fusion protein (DCC-C) and Nck-1 was assessed using a dot blot assay. In brief, GST (5 or 50 μg), GST-p65 MAPK (5 μg), GST-CdGAP proline-rich domain (5 μg), and GST-DCC-C (5 μg) were spotted onto nitrocellulose membranes. The filter was air-dried and incubated with blocking buffer (Tris-buffered saline containing 5% milk and 0.05% Tween 20) for 2 h at room temperature. The cDNA encoding Nck-1 was subcloned into pGEX-2TK (Amersham Biosciences) containing a pro-tein kinase site located between the GST domain and the multi-cloning site that allows expression of the full length protein. The GST-Nck-1 fusion protein was expressed in E. coli and purified using glutathione-Sepharose beads at 4 °C. The GST-Nck-1 fusion protein was then incubated with blocking buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 150 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM glutathione. The membrane was incubated overnight at 4 °C in kinase buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DCC-C for 2 h. Beads were washed three times with cold PBS, and radiolabeled GST-Nck-1 was eluted in Buffer D (50 mM Tris, pH 8, 150 mM NaCl, 5 mM MgCl₂, 1 mM glutathione-Sepharose beads at 4 °C overnight. The membrane was washed with Tris-buffered saline containing 5% milk and 0.05% Tween 20) for 2 h at room temperature. The GST fusion proteins were visualized by autoradiography.

**Summary of the binding of DCC to the various Nck-1 protein mutants and their effect on DCC-induced neurite outgrowth**

| Nck¹⁻³⁷⁷ | Nck-(SH₃/SH₂/SH₁)¹⁻²⁵¹ | Nck¹⁻⁶⁵ | Nck¹⁻⁸⁵⁻²⁶⁶ | Nck¹⁻¹⁰⁸⁻¹⁶⁵ | Nck¹⁻¹⁰⁸⁻¹⁶⁵ | Nck¹⁻¹⁰⁸⁻¹⁶⁵ | Nck¹⁻¹⁰⁸⁻¹⁶⁵ |
|---------|----------------------|---------|---------------|---------------|---------------|---------------|---------------|
| Nck¹⁻³⁷⁷ | Nck¹⁻³⁷⁷ | Nck¹⁻³⁷⁷ | Nck¹⁻³⁷⁷ | Nck¹⁻³⁷⁷ | Nck¹⁻³⁷⁷ | Nck¹⁻³⁷⁷ | Nck¹⁻³⁷⁷ |

**RESULTS**

**The Netrin-1 Receptor DCC Interacts with Nck in Embryonic Spinal Commissural Neurons Independently of Netrin-1**—To...
determine whether Nck plays a role in the netrin-1 receptor DCC signaling, we attempted to determine if endogenous Nck and DCC could be co-precipitated from rat day 1 brain lysate. In this experiment, DCC was found to co-precipitate with Nck in rat whole brain protein lysates, with no band corresponding to DCC observed in immunoprecipitates using normal mouse IgGs (Fig. 1A). To assess whether the interaction between endogenous Nck and DCC is similar in neurons that respond to netrin-1, we immunoprecipitated Nck from dissociated commissural neurons obtained from the dorsal half of micro-dissected embryonic day 13 rat spinal cords. After 2 days in culture, cells were incubated with or without 160 ng/ml netrin-1 for 10 min before cell lysis. DCC was found to co-precipitate with Nck both in the absence or in the presence of netrin-1, and DCC was not significantly detected in the negative control using normal mouse IgGs (Fig. 1B). After netrin-1 treatment, the higher amount of DCC proteins co-precipitated with Nck correlated with the increased amount of immunoprecipitated Nck (Fig. 1D). Time course incubation with netrin-1 from 5 to 20 min did not show any increase in the level of DCC interacting with Nck (data not shown). Furthermore, when DCC was co-expressed with Nck-1 in COS-7 cells that do not express endogenous DCC or netrin-1 (15), DCC also co-immunoprecipitated with Nck-1 in the absence of netrin-1 (Fig. 1C).

As previously found (16), DCC was observed in the cell bodies along the axons of the commissural neurons and as a punctate staining enriched in the growth cones and at the tip of the filopodia (Fig. 2, A and B). Nck was also highly enriched in the cell bodies and along the axons. Similar to the distribution of DCC, Nck was present in the filopodia and lamellipodia of the growth cones (Fig. 2, A and B). Confocal microscopy analysis revealed that DCC and Nck highly colocalized in the cell bodies and along the axons both in the presence of netrin-1 (Fig. 2B) and in the absence (Fig. 2A) of netrin-1. Hence, DCC interacts with Nck in vivo, and this interaction is independent of netrin-1 in embryonic commissural neurons.

Nck-1 Binds Directly to the Cytoplasmic Tail of DCC through the First and Third SH3 Domains—To characterize the interaction of DCC with Nck, GST pull-down experiments were performed using lysates of COS-7 cells expressing DCC incubated with wild type or mutated Nck-1 expressed as GST fusion proteins (Table I). As shown in Fig. 3A, wild type Nck-1 or the three SH3 domains of Nck-1 showed binding to DCC, whereas the Nck-1 SH2 domain showed no interaction. To dissect the interaction of DCC with the SH3 domains of Nck-1, each of the SH3 domains of Nck-1 were expressed as GST fusion proteins
Nck-1 Binds to the Netrin-1 Receptor DCC

Nck-1 Couples the Netrin-1 Receptor DCC to Activation of Rac1 in Swiss 3T3 Fibroblasts—Stimulation of DCC receptors with netrin-1 triggers actin reorganization through activation of Rac1 but not Cdc42 or RhoA in Swiss 3T3 fibroblasts (15).

**Nck-1 Binds to the Netrin-1 Receptor DCC**

**A**

![Graph showing percentage of transfected cells with neurite outgrowth](image)

**B**

![Western blot](image)

**Fig. 4. A dominant negative Nck-1 inhibits DCC-induced neurite outgrowth in N1E-115 cells.** A, N1E-115 neuroblastoma cells were transfected either with pRK5, pRK5-DCC or pcDNAmyc-Nck(1-377), -Nck(308K)1-377, -Nck(W38R,W143R,W229R)1-377, or -Nck-(SH3/SH3/SH3)1-251 alone or with pRK5-DCC and pcDNAmyc, encoding the various Nck-1 protein mutants. Values indicate the percentage of transfected cells with neurite extension and correspond to the average of at least three independent experiments. B, total cell lysates were submitted to SDS-PAGE, and levels of DCC and wild type or mutant Nck-1 proteins were detected by Western blotting using monoclonal anti-DCC and polyclonal anti-Nck antibodies, respectively.

and incubated with lysates of DCC-expressing cells. The third SH3 domain of Nck-1 was found to interact with DCC. In addition, the first SH3 domain of Nck-1 also bound to DCC but to a lower extent. No significant binding was observed with the second SH3 domain (Fig. 3A). Full-length Nck-1 containing amino acid substitutions W38R, W143R, and W229R in each of the SH3 domains predicted to inhibit interactions with proline-containing proteins (30, 31) did not interact with DCC (Fig. 3A). However, Nck-1 protein mutants containing these amino acid substitutions in individual SH3 domains were still able to interact with DCC (Fig. 3B). These results suggest that the first and third SH3 domains of Nck-1 are sufficient to mediate the interaction with DCC.

To assess the specificity of the interaction between DCC and Nck-1, we incubated lysates of DCC-expressing cells with the SH3 domains of Grb-2, Src, and the endocytic protein intersectin. As shown in Fig. 3C, no binding was observed with the carboxyl-terminal SH3 domain of Grb2, the SH3 domain of Src, or the SH3A and -D domains of intersectin, suggesting a specific interaction between Nck-1 and DCC. To determine whether Nck-1 interacts directly with the cytoplasmic tail of DCC, we have expressed a GST fusion protein with the complete intracellular domain of DCC (DCC-C) in *E. coli*. Direct binding between DCC-C and Nck-1 was assessed in a dot blot assay using purified Nck-1 expressed as a GST fusion protein and phosphorylated in *vitro* with [γ-32P]ATP (Fig. 3D). Nck-1 was able to bind directly to the cytoplasmic domain of DCC and to the serine/threonine kinase p65 PAK known to interact with the second SH3 domain of Nck-1 (32, 33). No binding was observed with the proline-rich domain of CdGAP or with high amounts of GST proteins. Therefore, Nck-1 interacts directly in *vitro* with the intracellular domain of DCC.

**The SH2 Domain of Nck-1 Is Required for DCC to Induce Neurite Outgrowth in N1E-115 Neuroblastoma Cells**—Mouse N1E-115 neuroblastoma cells express netrin-1 but not DCC (15). When DCC is ectopically expressed in N1E-115 cells, the majority of DCC-expressing cells show neurite outgrowth in a netrin-1-dependent manner (15). To address the cellular function of Nck-1 in the context of netrin-1-mediated DCC signaling, we expressed full-length Nck-1 or various Nck-1 mutant proteins alone or together with DCC into N1E-115 cells and measured the number of transfected cells with neurite outgrowth (Fig. 4A and Table I). 60% of transfected cells with the expression vector pRK5 encoding DCC exhibited neurite outgrowth, whereas Nck-1 induced neurite outgrowth in more than 50% of transfected cells. When DCC and Nck-1 were expressed together in N1E-115 cells (Fig. 4B), 80% of transfected cells now showed neurite outgrowth (Fig. 4A). When full-length Nck-1 containing a R308K substitution to block the interaction between the SH2 domain of Nck-1 and phosphoryrosine-containing proteins (30, 31) was expressed in N1E-115 cells, it was unable to induce neurite outgrowth. This suggested that the SH2 domain is required for Nck-1 to induce neurite outgrowth in N1E-115 cells. Similarly, a deletion mutant protein of Nck-1 lacking the SH2 domain was unable to induce neurite outgrowth. When DCC was expressed in the presence of Nck-1 mutant proteins containing a R308K substitution or lacking the SH2 domain, the formation of neurite outgrowth was almost completely abolished. Alternatively, Nck-1 containing the W38R, W143R, and W229R substitutions in each of the SH3 domains was unable to induce neurite outgrowth but did not interfere in the formation of neurite outgrowth provoked by the expression of DCC in N1E-115 cells. Therefore, the SH3 and SH2 domains of Nck-1 are required for Nck-1 to induce neurite outgrowth in N1E-115 cells. Furthermore, Nck-1 lacking a functional SH2 domain acts as a dominant negative protein by interacting with DCC but is unable to mediate the downstream signaling pathways, leading to induction of neurite outgrowth. Thus, the SH2 domain of Nck-1 plays an essential role in mediating neurite outgrowth induced by DCC.

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**Neurite Outgrowth in N1E-115 Neuroblastoma Cells**—When DCC is ectopically expressed in N1E-115 cells, the majority of DCC-expressing cells show neurite outgrowth in a netrin-1-dependent manner (15). To address the cellular function of Nck-1 in the context of netrin-1-mediated DCC signaling, we expressed full-length Nck-1 or various Nck-1 mutant proteins alone or together with DCC into N1E-115 cells and measured the number of transfected cells with neurite outgrowth (Fig. 4A and Table I). 60% of transfected cells with the expression vector pRK5 encoding DCC exhibited neurite outgrowth, whereas Nck-1 induced neurite outgrowth in more than 50% of transfected cells. When DCC and Nck-1 were expressed together in N1E-115 cells (Fig. 4B), 80% of transfected cells now showed neurite outgrowth (Fig. 4A). When full-length Nck-1 containing a R308K substitution to block the interaction between the SH2 domain of Nck-1 and phosphoryrosine-containing proteins (30, 31) was expressed in N1E-115 cells, it was unable to induce neurite outgrowth. This suggested that the SH2 domain is required for Nck-1 to induce neurite outgrowth in N1E-115 cells. Similarly, a deletion mutant protein of Nck-1 lacking the SH2 domain was unable to induce neurite outgrowth. When DCC was expressed in the presence of Nck-1 mutant proteins containing a R308K substitution or lacking the SH2 domain, the formation of neurite outgrowth was almost completely abolished. Alternatively, Nck-1 containing the W38R, W143R, and W229R substitutions in each of the SH3 domains was unable to induce neurite outgrowth but did not interfere in the formation of neurite outgrowth provoked by the expression of DCC in N1E-115 cells. Therefore, the SH3 and SH2 domains of Nck-1 are required for Nck-1 to induce neurite outgrowth in N1E-115 cells. Furthermore, Nck-1 lacking a functional SH2 domain acts as a dominant negative protein by interacting with DCC but is unable to mediate the downstream signaling pathways, leading to induction of neurite outgrowth. Thus, the SH2 domain of Nck-1 plays an essential role in mediating neurite outgrowth induced by DCC.
Expression of DCC stimulates the formation of lamellipodia and membrane ruffles after the addition of netrin-1 for 10 min in a Rac1-dependent effect. To determine whether Nck-1 mediates the activation of Rac1 by DCC, we co-injected the expression vector pRK5 encoding full-length DCC alone or together with dominant negative Nck-1 containing the three SH3 domains expressed as a GST fusion protein into quiescent, serum-starved Swiss 3T3 cells. As expected, in the presence of netrin-1, DCC induced the formation of lamellipodia and stress fibers (Fig. 5C). In contrast, the presence of dominant negative Nck-1 inhibited the effect of DCC on actin, whereas the presence of GST proteins had no effect on the formation of lamellipodia in cells expressing DCC (Fig. 5, E and G). To confirm that the inhibitory effect on Rac1 activation is specific to the SH3 domains of Nck-1, we co-injected DCC-encoding vector together with the SH3 domain of Grb2 expressed as a GST fusion protein into Swiss 3T3 fibroblasts. As shown in Fig. 5I, Grb2-SH3 previously shown not to bind to DCC (Fig. 3C) was unable to block the formation of lamellipodia induced by the netrin-1 receptor DCC. Therefore, we propose that the interaction of Nck-1 with DCC is required for Rac1 activation, leading to actin reorganization in fibroblasts. These results also demonstrate that the SH2 domain of Nck-1 is necessary to mediate the activation of Rac1 by DCC in the presence of netrin-1.

**DCC Associates with the Actin Cytoskeleton and Re-localizes Nck-1 to the Triton-insoluble Fraction in a Ligand-independent Manner**—DCC expressed in Swiss 3T3 fibroblasts in the presence of netrin-1 was found to cluster within lamellipodia and membrane ruffles after the addition of netrin-1 for 10 min in a Rac1-dependent effect. To determine whether Nck-1 mediates the activation of Rac1 by DCC, we co-injected the expression vector pRK5 encoding full-length DCC alone or together with dominant negative Nck-1 containing the three SH3 domains expressed as a GST fusion protein into quiescent, serum-starved Swiss 3T3 cells. As expected, in the presence of netrin-1, DCC induced the formation of lamellipodia and stress fibers (Fig. 5C). In contrast, the presence of dominant negative Nck-1 inhibited the effect of DCC on actin, whereas the presence of GST proteins had no effect on the formation of lamellipodia in cells expressing DCC (Fig. 5, E and G). To confirm that the inhibitory effect on Rac1 activation is specific to the SH3 domains of Nck-1, we co-injected DCC-encoding vector together with the SH3 domain of Grb2 expressed as a GST fusion protein into Swiss 3T3 fibroblasts. As shown in Fig. 5I, Grb2-SH3 previously shown not to bind to DCC (Fig. 3C) was unable to block the formation of lamellipodia induced by the netrin-1 receptor DCC. Therefore, we propose that the interaction of Nck-1 with DCC is required for Rac1 activation, leading to actin reorganization in fibroblasts. These results also demonstrate that the SH2 domain of Nck-1 is necessary to mediate the activation of Rac1 by DCC in the presence of netrin-1.
co-localize with regions enriched in polymerized actin (Fig. 6C). COS-7 cells expressing DCC in the presence or absence of netrin-1 for 30 min were extracted using Triton X-100, and proteins present in the detergent-soluble and -insoluble fractions were identified by immunoblotting. 32% of DCC was resistant to extraction with 0.4–1.0% Triton X-100 in cells either treated or untreated with netrin-1 (Fig. 7, A, B, and D). The cells were processed under identical conditions, and Rac1 was revealed using an anti-myc antibody. D, the level of DCC and Nck-1 proteins present in the Triton X-100-insoluble and -soluble fractions after treatment of cells with 1% Triton X-100 was estimated by densitometry.

DISCUSSION

Axon guidance results from coordinated cell movements in which the neuronal growth cone receives signals from the environment and translates them into changes in the actin- and microtubule-based cytoskeleton that lead ultimately to directed neurite outgrowth (1). Growth cone movements require the expression of axon guidance receptors responsive to extracellular guidance cues with attractive or repulsive signals specifying the direction of axon outgrowth. Recent studies demonstrate a role for the cytoplasmic domains of transmembrane guidance receptors and the implication of the Rho GTPases to effect changes in motility (4). Furthermore, the small GTPases Rac1, Cdc42, and RhoA act as essential components in the cytosolic signaling mechanisms induced by DCC when it binds to netrin-1 (15, 16). The cytoplasmic domain of DCC is essential for neurite outgrowth in N1E-115 cells and to activate Rac1 in fibroblasts (15).

A role for the SH3/SH2 adaptor Nck-1 in netrin-1-mediated DCC signaling is now indicated. Nck and DCC physically interact in newborn rat brains and in commissural neurons from E13 rat spinal cord. DCC and Nck colocalize in the cell bodies as well as in the axons and growth cones of commissural neurons. The association of DCC and Nck was present in the absence of netrin-1 both in commissural neurons and in COS-7 cells expressing DCC in the presence or absence of netrin-1. Thus, these results provide further evidence that the netrin-1 receptor DCC interacts with Nck-1 in the same cellular compartment coincident with actin changes and axon outgrowth.
cells overexpressing DCC and Nck-1. The *in vitro* interaction between Nck-1 and DCC is direct and mediated by the first and third SH3 domains of Nck-1. Therefore, the interaction between the SH3 domains of Nck-1 with putative PXXP motifs in the receptor DCC is likely to be constitutive. This is not surprising since most, but not all, SH3-PXXP interactions are often constitutive, whereas SH2-Tyr(P) binding is always induced by extracellular signals (23).

A role for Dock, the fly homologue of mammalian Nck, in axon guidance is well documented (23). During a genetic screen to identify effectors involved in axonal pathfinding, Dock was identified as an essential gene for proper photoreceptor axon targeting and fasciculation (18). In addition to its role in the adult fly visual system, Dock has been shown to play an essential role in synapse formation in the embryonic nervous system. Dock protein is expressed in most or all central nervous system axons and cell bodies (21). Interestingly, Dock and Frazzled, the fly homologue of DCC, are both expressed on commissural and longitudinal axons in the developing central nervous system (8, 21). Whether Dock and Frazzled interact together in the fly system is not known and will be of great interest to demonstrate. Recently, Dock was shown to interact with the axon guidance receptors, Dscam and Kette (19, 20). Dock directly interacts with Dscam through both its SH2 and SH3 domains. Genetic studies showed that Dscam, Dock, and the Rac/Cdc42 effector p65PAK act together to direct axon pathfinding (19). Similar to its mammalian counterpart, HEM-2/NAP, which has been shown to interact with the first SH3 domain of Nck-1 (34), Kette and Dock interact genetically (20). Whether Dock interacts constitutively with Dscam or Kette is not known and will have to await the identification of the ligands to understand better the precise mechanisms.

An essential role for the SH2 domain of Nck-1 in mediating DCC-induced neurite outgrowth in N1E-115 cells and the activation of Rac1 in Swiss 3T3 cells was demonstrated here. The expression of the three SH3 domains of Nck-1 blocked the ability of DCC to induce neurite outgrowth in N1E-115 cells or to induce lamellipodia in the presence of netrin-1 in fibroblasts. Whereas the first and third SH3 domains of Nck-1 are required to bind to DCC, the SH2 domain appears to be functionally essential to mediate the immediate downstream signaling mechanisms induced by the netrin-1 receptor DCC. Studies in mammalian systems demonstrate that Nck-1 links cell surface tyrosine kinase receptors through its SH2 domain to downstream effectors via the SH3 domains (23). Here, we propose an atypical "coupling mechanism" in which Nck-1 links the growth cone guidance receptor DCC via SH3 domains to intracellular effectors through its SH2 domain, leading to activation of Rac1 (Fig. 8). This coupling mechanism may represent a general mechanism of action of Dock/Nck-1 coupled to axon guidance receptors including Frazzled/DCC, Dscam, and kette/HEM-2/NAP in *Drosophila melanogaster* and in mammalian tissues.

DCC associates constitutively with the actin cytoskeleton. In the absence of DCC, most of Nck-1 was found in the soluble fraction, whereas the presence of DCC relocalized 51% of Nck-1 into the Triton-insoluble fraction. Therefore, in the absence of ligand, DCC may be responsible for the association of Nck-1 with the underlying actin cytoskeleton. DCC binding to netrin-1 may result in a conformational change of Nck-1 that is now able to interact with specific effectors via its SH2 domain to promote neurite outgrowth and activation of Rac1 (Fig. 8). The formation of a DCC/Nck-1 molecular complex associated with the underlying actin cytoskeleton is likely to be a major step involved in growth cone motility and guidance in response to a source of netrin-1.

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