p130\textsuperscript{CAS} Is Required for Netrin Signaling and Commisural Axon Guidance

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Netrins are an important family of axon guidance cues. Here, we report that netrin-1 induces tyrosine phosphorylation of p130\textsuperscript{CAS} (Crk-associated substrate). Our biochemical studies indicate that p130\textsuperscript{CAS} is downstream of the Src family kinases and upstream of the small GTPases Rac1 and Cdc42. Inhibition of p130\textsuperscript{CAS} signaling blocks both the neurite outgrowth-promoting activity and the axon attraction activity of netrin-1. p130\textsuperscript{CAS} RNA interference inhibits the attraction of commissural axons in the spinal cord by netrin-1 and causes defects in commissural axon projection in the embryo. These results demonstrate that p130\textsuperscript{CAS} is a key component in the netrin signal transduction pathway and plays an important role in guiding commissural axons in vivo.

Key words: netrin-1; p130\textsuperscript{CAS}; Rac1; Cdc42; signaling; commissural axons

Introduction

Netrins are a family of secreted proteins that can both promote axon outgrowth and guide growth cone navigation in species ranging from Caenorhabditis elegans to mammals (Tessier-Lavigne et al., 1988; Hedgcock et al., 1990; Ishii et al., 1992; Kennedy et al., 1994; Serafini et al., 1994; Kolodziej et al., 1996; Mitchell et al., 1996). The receptors for uncoordinated protein 6 (UNC-6)/netrin were uncovered in C. elegans as UNC-40 and UNC-5 (Leung-Hagesteijn et al., 1992; Chan et al., 1996). The mammalian homologs of UNC-40 are deleted in colorectal cancer (DCC) and neogenin (Keino-Masu et al., 1996; Fazeli et al., 1997). DCC contains a large extracellular domain, a single transmembrane domain, and a cytoplasmic region with three conserved domains, P1, P2, and P3 (Chan et al., 1996; Keino-Masu et al., 1996). Our studies of signal transduction mechanisms mediating netrin attraction have focused on events downstream of DCC.

We and others have shown recently that the P3 domain of DCC interacts with the Src family tyrosine kinase Fyn and the focal adhesion kinase (FAK) and that these kinases are essential for attractive signaling by netrin (Li et al., 2004; Liu et al., 2004; Ren et al., 2004). Previous studies have found that netrin activates the small GTPases Rac1 and Cdc42 (Li et al., 2002; Shekarabi and Kennedy, 2002). However, the small GTPases can be downstream of a number of molecules, whereas FAK can regulate a fair number of molecules, including the phospholipase C (PLC)-γ, phosphoinositol-3 (PI3)-kinase, Akt, mitogen-activated protein (MAP) kinases, paxillin, p130\textsuperscript{CAS}, Crk, and Graf (Parsons et al., 2000; Schaller, 2001; Hanks et al., 2003). Therefore, what functions downstream of FAK in netrin signaling is not clear.

p130\textsuperscript{CAS} [Crk-associated substrate (Cas)] was first identified as a highly phosphorylated protein in cells transformed by v-crk (Matsuda et al., 1990; Birge et al., 1992; Sakai et al., 1994) and v-src (Reynolds et al., 1989; Kanner et al., 1991; Sakai et al., 1994) oncogenes. It binds to multiple cellular proteins and is involved in a variety of biological processes, including cell adhesion, cell migration, growth factor stimulation, cytokine receptor engagement, bacterial infection, cell proliferation, and survival (for review, see O’Neill et al., 2000; Bouton et al., 2001). p130\textsuperscript{CAS} knockout mice are embryonically lethal with cardiovascular defects (Honda et al., 1998). p130\textsuperscript{CAS} has multiple protein–protein interaction motifs, including an Src-homology 3 (SH3) domain, a proline-rich segment, a YXXP domain (substrate-binding domain) containing 15 repeats of a four amino acid sequence (tyrosine-X-X-proline), a serine-rich region, and a C-terminal domain (for review, see O’Neill et al., 2000; Bouton et al., 2001). Tyrosine residues in p130\textsuperscript{CAS} are functionally important for cell migration and biochemically phosphorylated by Src (Manie et al., 1997; Ruest et al., 2001; Brabek et al., 2005).

We report here that p130\textsuperscript{CAS} is a key component in the netrin attraction pathway, functioning downstream of Fyn and FAK and upstream of Rac1, providing a link from the tyrosine kinases to one of the small GTPases.
Materials and Methods

We used the following antibodies: anti-Fyn, anti-actin, anti-p130Cas, and anti-phospho-FAK (Santa Cruz Biotechnology, Santa Cruz, CA); CAS phosphotyrosines 165, 249, and 410 antibodies (Cell Signaling Technology, Beverly, MA); anti-FAK (Santa Cruz Biotechnology; Transduction Laboratories, Lexington, KY); anti-Cdc42, anti-Rac1, and anti-DCC (BD Biosciences, Franklin Lakes, NJ); anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY); anti-phospho-Src tyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY); anti-hemagglutinin (HA) (Covance, Princeton, NJ); and anti-axonin-1 (gift from E. T. Stoeckli, University of Zurich, Zurich, Switzerland); and anti-p130 Cas for immunohistochemistry (gift from R. Sakai, National Cancer Research Institute, Tokyo, Japan). Plasmids encoding DCC-Delta1 (Δ1147–1170), ΔP2 (Δ1335–1356), ΔP3 (Δ1412–1447), 1/2 ΔP3 (Δ1426–1447); Y1420F; and 4Y/F (Y1261F, Y1272F, Y1363F, Y1420F) have been described previously (Li et al., 2004). p130Cas (F15) is a mouse p130Cas mutant, in which all 15 YXXP tyrosines in the substrate domain have been mutated to phenylalanines (Shin et al., 2004).

The targeted sequence of short hairpin-based RNA (shRNA) construct of p130Cas is: GACATCTACCAAGTTCCTC. The human U6 promoter is known to function in chickens (Bron et al., 2004; Dai et al., 2005).

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Netrin-1 protein was purified with anti-myc tag affinity matrix from the conditioned media of human embryonic kidney 293 (HEK293) cells stably secreting netrin-1. The control was made by sham purification from the conditioned media from HEK293 cells that had not been transfected with a cDNA expressing the myc-tagged netrin-1.

**Dissociated primary neuron cultures.** The dissociated culture procedure was done as described previously (Liu et al., 2004) with some modifications. Briefly, embryos were removed from freshly killed pregnant mice of the appropriate stage. The brain or the spinal cord was dissected in cold HBSS medium (Invitrogen, San Diego, CA), and meninges were removed. The cortices or spinal cords were cut into small pieces with scissors and trypsinized at 37°C for 15 min. After trituration several times, cells were resuspended in DMEM supplemented with heat-inactivated fetal calf serum (Invitrogen) and 20 U/ml of penicillin/streptomycin. Cells were grown on the poly-D-lysine (Sal) coated dishes at 37°C in a 5% CO2 incubator overnight.

For immunocytochemistry of p130Cas and DCC in dissociated primary neurons, cells were fixed for 10 min in 4% prewarmed paraformaldehyde solution (127 mM NaCl, 5 mM KCl, 1.1 mM Na2HPO4, 0.4 mM KH2PO4, 2 mM MgCl2, 5.5 mM glucose, 1 mM EGTA, 10 mM PIPES) and permeabilized in 0.5% Triton X-100 for 15 min after washed three times with PBS. Cells were blocked in the blocking buffer (PBS containing 3% BSA and 0.1% Triton X-100) at room temperature for 1 h and incubated with the anti-p130Cas antibody (rabbit, 1:200) and the anti-DCC antibody (mouse, 1:1000) at 37°C for 1 h. After being washed on coverslips in 1 × PBS three times, cells were incubated with the secondary antibodies (anti-rabbit-Cy3, 1:200; anti-mouse-Cy2, 1:200) at 37°C for 1 h. Images were taken under a confocal microscope.

For analysis of neurite outgrowth, cortical neurons were isolated from E15 mouse embryos and dissociated. Neurons (4 × 10^5/group) were mixed with Venus yellow fluorescent protein (YFP) (1 μg) plus control vector (4 μg) or p130Cas shRNA construct (4 μg) and immediately placed in Nucleofector (Amaxa Biosystems, Gaithersburg, MD).
within purified netrin-1 (250 ng/ml) or with the sham-purified control. Cells were then fixed with 4% PFA for 20 min and stained with phallolidin (Invitrogen, Eugene, OR). Nuclei were visualized with Hoechst dye.

**Immunoprecipitation and Western blot analysis.** Cytosolic cells were lysed with a modified radiolabeled immunoprecipitation assay buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% deoxycholic acid, 0.5% Triton X-100, 1 mM PMSF, 1 mM sodium orthovanadate, 1X protease inhibitor mixture (Roche Molecular Biochemicals)] as described previously (Liu et al., 2004). Lysates were immunoprecipitated with specific antibodies and protein A/G-agarose beads for 3 h or overnight at 4°C. For the immunoblotting, the washed immunoprecipitates were boiled in 1X SDS sample buffer for 5 min.

For immunoprecipitation of HEK cell lines, HEK293 cells were transfected with the Lipofectamine (Invitrogen) method. Cells were lysed 48 h after transfection in mild lysis buffer (MLB) (20 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1% NP-40, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) and followed by incubation with specific antibodies for 2 h before protein A/G-agarose beads were added at 4°C.

For Western blot analysis, protein extracts were separated with 7.5% SDS-PAGE. Western blots were visualized with the enhanced chemiluminescence kit (GE Healthcare, Arlington Heights, IL).

**Rac1 and Cdc42 activity assays.** Two × 10^5 HEK293 cells stably expressing DCC were transfected with the wild-type p130Cas or p130Cas (F15) constructs (5 μg/group) by the calcium phosphate method. Forty-eight hours after transfection, cells were stimulated with purified netrin-1 (500 ng/ml) or the sham-purified control for 5 min. After being washed with 1X PBS once, cells were lysed with MLB lysis buffer (25 mM HEPES, pH 7.5, 1% NP-40, 150 mM NaCl, 10% glycerol, 1 mM EDTA, and 10 mM MgCl2 containing protease inhibitor mixture) and centrifuged at 15,000 × g at 4°C for 15 min. The levels of active GTP-bound GT-Pase (Rac1, Cdc42) were measured as follows: 30 μg of the GST-Cdc42/Rac-interactive binding domain of p21-activated kinases (PAK) (CRIB) of PAK was coupled to glutathione-Sepharose beads (GE Healthcare) at 4°C for 30 min. These beads were used to pull down GT-Pase bound forms of Rac1 and Cdc42. The resulting supernatants after centrifugation were incubated with Sepharose bead-associated GST-PAK for 45 min at 4°C. The beads were washed three times with the lysis buffer, and the bound small GTPase proteins were separated by 15% SDS-PAGE. Western blot analysis was performed with rabbit anti-Rac1 and anti-Cdc42 antibodies (Wong et al., 2001).

**Commisural axon turning assay.** White leghorn chicken embryos were collected and staged according to Hamburger and Hamilton (1951). The electroporation procedure was essentially done as described previously (Liu et al., 2004). DNA solution was injected into the central canal of the neural tube of chick embryos *in ovo* at stages 12–15. The electroporation program was 25 V, 5 ms, 5 pulses (ECM830; BTX Instrument Division of...
Genetronics, San Diego, CA). Embryos were isolated at stages 20–21 and examined for YFP fluorescence under the microscope. The half of the spinal cord showing fluorescence was isolated as an open-book preparation and cocultured with an aggregate of control or netrin-1 secreting HEK cells for 24 h. Axon turning was counted when the angle of turning toward the HEK aggregate was >5°. The percentage of turning axons was calculated from the numbers of fluorescent axons turning toward the HEK cell aggregate divided by the total numbers of fluorescent axons within 300 μm of the HEK cell aggregates. Images were collected with a confocal microscope.

Analysis of commissural axon projection in vivo. Collection of chick embryos and electroporation procedures were described above. Chick embryos were killed between stages 22 and 23 after electroporation. This stage was chosen for analysis, because it is the time when the commissural axons in the lumbosacral region cross the midline (Stoeckli et al., 1997; Bourikas et al., 2005). The lumbosacral region of the spinal cord was isolated and the fluorescence examined under a microscope. Samples expressing green fluorescent protein (GFP) were chosen for opening at the roof plate (open-book preparation). Whole-mount immunostaining of the spinal cord was performed after fixation. Briefly, samples were permeabilized in 0.5% Triton for 15 min and blocked for 30 min in the blocking buffer (PBS containing 5% goat serum and 0.1% Triton X-100) at room temperature. Tissues were incubated with the first antibody (rabbit, anti-axonin-1, 1:1000) in the blocking buffer at 37°C for 2 h. After being rinsed with 1× PBS, tissues were incubated with the second antibody (anti-rabbit-Cy3 antibody, 1:200) at 37°C for 2 h. After being rinsed with PBS three times, the spinal cord in the open-book preparation was mounted in Gel/Mount (Biomeda, Foster City, CA). Images were taken under the confocal microscope. The percentage of axons reaching the floor plate was quantified from the numbers of fluorescently labeled commissural axons arriving at or crossing over the floor plate divided by the total numbers of fluorescent axons within 100 μm from the floor plate.

The lumbosacral region of the spinal cord expressing GFP at stage 23 was collected. Transverse sections of 200 μm were cut and mounted in Gel/Mount. Images were also taken under the confocal microscope.

In vivo electroporation of pregnant mice was performed as described previously (Saba et al., 2003) with minor modifications. The embryonic day 10.5 (E10.5) pregnant mice were anesthetized by intraperitoneal injection of 10% Nembutal solution. The uteri were cut and the uterus was carefully taken out. The DNA solution was injected into the central canal of the spinal cord. The electroporation program was 22 V, 5 ms, 5 pulses (BTX, ECM830). The uteri were carefully put back into the abdominal cavity, and the abdominal wall and skin were sewn up. The mice were killed 2 d later at stage E12.5 and fixed in 4% paraformaldehyde solution. Sections of embryos spinal cord (200 μm) were transversely cut and mounted in Gel/Mount. The fluorescent images were also taken under the confocal microscope.

**Results**

Netrin-1 stimulates tyrosine phosphorylation of p130CAS and its association with FAK and Fyn

We used antibodies for p130CAS and DCC to examine whether p130CAS is expressed in axons and growth cones containing DCC.
We found coexpression of p130CAS with DCC in the soma and cytoplasmic membrane as well as the axons, axonal branches, and the growth cones of primary neurons from the neocortex of E15 mice (Fig. 1a–f). Confocal analysis indicates partial colocalization of p130CAS with DCC (Fig. 1c,f). Similar observations were made when anti-p130CAS and anti-DCC antibodies were used to examine their localization in primary neurons from the dorsal spinal cord of E13 mouse embryos (Fig. 1g–m). Immunohistochemistry staining showed that p130CAS was strongly expressed in commissural axons of E13 embryonic spinal cord (Fig. 1n–o).

We observed that netrin-1 increased the phosphorylation of proteins of ∼130 kDa, one of which was FAK (Liu et al., 2004). Antibody depletion experiments showed that FAK was not the only protein in that band (Liu et al., 2004). To determine whether p130CAS is among them, we examined the p130CAS tyrosine phosphorylation after netrin-1 stimulation of dissociated E15 primary cortical neurons. We either immunoprecipitated extracts with the anti-p130CAS antibody and probed the Western blots with the anti-phosphotyrosine antibody or reversely immunoprecipitated with the anti-phosphotyrosine antibody and probed the Western blots with the anti-p130CAS antibody. Results from both procedures showed that tyrosine phosphorylation of p130CAS was induced by netrin-1 within 5 min (Fig. 2a,b). To directly detect the tyrosine phosphorylation of p130CAS, we used antibodies recognizing phosphotyrosines at residues 165 and 249 of p130CAS. Netrin-1 increased tyrosine phosphorylation at both sites of p130CAS in E15 primary cortical neurons (Fig. 2c) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) and in dissociated E13 dorsal spinal cord neurons (Fig. 2d,e). Induction of tyrosine phosphorylation in p130CAS was inhibited by a function-blocking antibody against DCC (Fig. 2d).

p130CAS is known to form a protein–protein interaction complex with the Src family kinases and FAK. Because netrin-1 activates Fyn and FAK (Li et al., 2004; Liu et al., 2004; Ren et al., 2004), we performed immunoprecipitation experiments to test whether netrin-1 regulated the interaction of p130CAS with Fyn and FAK. Netrin-1 increased the interaction of p130CAS with FAK (Fig. 2f) and Fyn (Fig. 2g) in E15 primary cortical neurons.

There are three conserved intracellular domains (P1, P2, and P3 in DCC), among which P3 is required for netrin-induced Fyn and FAK phosphorylation (Li et al., 2004; Ren et al., 2004). We investigated the domains of DCC involved in p130CAS tyrosine phosphorylation after netrin-1 stimulation. Deletion of P1 (residues 1147–1171) or P2 (residues 1335–1356) did not inhibit the induction of p130CAS phosphorylation by netrin-1 (Fig. 2h). However, deletion of P3 domain (residues 1412–1447) (Fig. 2h) or half of the P3 domain (residues 1426–1447) blocked the effect of netrin-1 on p130CAS phosphorylation (Fig. 2i). Furthermore, mutation of one tyrosine phosphorylation site (1420Y/F) or all of sites (1261Y/F, 1272 Y/F, 1363 Y/F, Y 1420 Y/F) of DCC intracellular domains had no effect on the induction of p130CAS tyrosine phosphorylation by netrin-1 (Fig. 2i). The domain in DCC required for p130CAS phosphorylation is similar to that for Src and FAK phosphorylation (Li et al., 2004).

p130CAS is downstream of FAK and Src but upstream of Rac1 and Cdc42

FAK and Src family kinases can be activated by netrin-1 (Li et al., 2004; Liu et al., 2004; Ren et al., 2004). To determine their relationship with p130CAS, we tested the effect of FAK and Src inhibition on p130CAS phosphorylation, as well as the effect of inhibition on FAK and Fyn phosphorylation.

To examine the role of FAK in netrin-induced p130CAS tyrosine phosphorylation, HEK293 cells were cotransfected with DCC, p130CAS tagged with the hemagglutinin epitope (HA-CAS), and a myc epitope tagged form of FRNK (FAK-related nonkinase), a dominant-negative FAK mutant containing the C-terminal noncatalytic region of FAK that can bind to FAK-interacting proteins but cannot function as an active kinase. p130CAS phosphorylation was assayed by immunoprecipitation with the anti-HA antibody and probed with the anti-phosphotyrosine antibody. Netrin-1 induced tyrosine phosphorylation of p130CAS in a DCC-dependent manner (Fig. 3a). The induction of p130CAS tyrosine phosphorylation by netrin-1 was inhibited by expression of FRNK (Fig. 3a), indicating that FAK is required for p130CAS phosphorylation. Netrin-induced p130CAS tyrosine phosphorylation was also inhibited by PP2, a pharmacological inhibitor of the Src family kinases, but not PP3, an inactive control for PP2 (Fig. 3a). Netrin induction of tyrosine phosphorylation of endogenous p130CAS in E15 neurons was also inhibited by PP2 (Fig. 3b). These findings indicate that netrin-induced tyrosine phosphorylation of p130CAS requires FAK and Src family kinases.

To determine whether netrin-induced FAK phosphorylation depends on p130CAS, we used two p130CAS mutants: p130CAS (F15), in which all of 15 tyrosine residues in the substrate binding domain were mutated to phenylalanine; and HA-CAS-SH3, which contains only the SH3 domain of p130CAS. p130CAS (F15)
These results indicate that p130CAS is required in the signaling trin activation of Rac1 and Cdc42 (Fig. 4). p130CAS (F15) blocked netrin-1-induced neurite outgrowth of cortical neurons cultured for 20 h. Both the length of the longest neurite from each neuron (l) and the total length of all neurites from each neuron (m) were inhibited by p130CAS shRNA. The p values are < 0.0001 between RNAi-treated neurons and control neurons. The length on the y-axis is in micrometers. k, Quantification of the total length of all neurites from each cortical neuron -axis is in micrometers. l, Quantification of the total length of all neurites from each cortical neuron after culturing for 20 h. Scale bar, 20 μm. m, Neurite outgrowth from YFP-positive neurons in the presence of purified netrin-1 (b, d) or the sham-purified control (a, c) after culturing for 20 h. Similar to a–d except that neurons were cultured for 40 h. Scale bar, 20 μm. i, j, Quantification of netrin-1-induced neurite outgrowth of cortical neurons cultured for 20 h. The difference is significant (p = 0.0001) between Groups II and IV. e–h, Similar to a–d except that neurons were cultured for 40 h. Scale bar, 20 μm. i, j, Quantification of netrin-1-induced neurite outgrowth of cortical neurons cultured for 20 h. The difference between Groups II and IV is very significant (p < 0.0001); the difference between Groups III and IV is not significant (p = 0.577). These results indicate that netrin promoted neurite outgrowth (compare Groups I and II) and that p130CAS shRNA significantly inhibited the effect of netrin (compare Groups II and IV). i, Quantification of the total length of all neurites from each cortical neuron cultured for 40 h. Group I, 3.52 ± 1.15 μm; Group II, 11.08 ± 5.44 μm; Group III, 56.67 ± 4.46 μm; Group IV, 53.31 ± 4.53 μm. 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transfected with the control vector (Fig. 5a,b), neurite outgrowth was stimulated by netrin-1: without netrin treatment, the length of the longest neurite of each neuron is 25.15 ± 1.26 μm, and the total length of all neurites from each neuron is 52.62 ± 2.27 μm;
with netrin treatment, the length of the longest neurite of each neuron is 56.91 ± 2.72 μm, and the total length of all neurites from each neuron is 90.04 ± 3.03 μm (Fig. 5i,j).

p130CAS shRNA inhibited netrin-induced neurite outgrowth: the length of the longest neurite per neuron was increased from 24.51 ± 1.52 μm without netrin to 34.13 ± 2.58 μm with netrin, and the total length of all neurites per neuron was increased from 54.06 ± 2.18 μm without netrin to 64.00 ± 2.98 μm with netrin (Fig. 5c,d,i,j). Comparing netrin-induced outgrowth from vector-transfected neurons (Fig. 5i,j, group II) to netrin-induced outgrowth from p130CAS shRNA-transfected neurons (Fig. 5i,j, group IV), the difference is statistically significant [p < 0.0001 between groups II and IV (Fig. 5i,j)]. Similar effects of p130CAS shRNA on netrin-induced neurite outgrowth were observed after 40 h of RNAi transfection (Fig. 5c–h,k,l). The neurite outgrowth promotion activity of netrin-1 was not blocked by the control shRNA (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Together, these results indicate that p130CAS is required for netrin-induced neurite outgrowth from primary neurons.

p130CAS is required for axon attraction by netrin-1

The commissural axons in the neural tube are a model for studying netrin attraction (Tessier-Lavigne et al., 1988; Kennedy et al., 1994; Serafini et al., 1994). To determine whether p130CAS is required for axon turning toward netrin-1, we used the open-book assay with commissural axons from the neural tube of chick embryos. As described previously (Liu et al., 2004), the Venus YFP construct was electroporated into the chick neural tube at stages 12–15. An explant of the neural tube was placed to one side of the open book. Projecting axons were visualized by Venus YFP expression. More than 90% of axons projecting from the dorsal spinal cord of chick embryos electroporated at these stages were commissural axons as confirmed by immunostaining with the anti-axonin-1 antibody (Figs. 8, 9) (supplemental Fig. S1, available at www.jneurosci.org as supplemental material).

Commissural axons projected straight toward the floor plate when the neural tube explants were cocultured with control HEK293 cells (Fig. 6a). Axons turned toward HEK cells secreting netrin-1 (Fig. 6b). Cotransfection of Venus YFP with either the vector or the wild-type p130CAS did not affect netrin attraction (Fig. 6d,f). However, when the dominant-negative mutant p130CAS (F15) was cotransfected into the commissural axons
together with Venus YFP, attraction by netrin was significantly inhibited (Fig. 6h,i).

To confirm the role of p130CAS, we introduced p130CAS shRNA or the vector shRNA into the neural tube by electroporation. When the vector shRNA was electroporated into the neural tube together with Venus YFP, commissural axons turned toward netrin-1 (Fig. 7b,i), whereas p130CAS shRNA cotransfected with Venus YFP significantly inhibited axon turning toward netrin

(7di)). Axon turning toward netrin was also inhibited by p130CAS siRNA (Fig. 7e,f,i). The effect of p130CAS siRNA on commissural axon turning could be rescued by the overexpression of wild-type p130CAS (Fig. 7g,h,i), indicating that the effect was specifically a result of p130CAS knockdown. Immunohistochemistry with the anti-axonin-1 antibody recognizing commissural axons revealed that only commissural axons transfected with p130CAS RNAi did not turn toward netrin, whereas untransfected axons were still attracted by netrin-1 (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

To test whether p130CAS knockdown by shRNA and siRNA caused the commissural axons to lose the turning ability, rather than only losing netrin responsiveness, we used the open-book preparation to assay for axon turning by the roof plate (supplemental Fig. 4a, available at www.jneurosci.org as supplemental material). As reported previously (Augsburger et al., 1999), the roof plate repelled the commissural axons (supplemental Fig. 4b,c, available at www.jneurosci.org as supplemental material). The repulsive response of commissural axons was not inhibited by p130CAS shRNA or siRNA (supplemental material). Thus, results from both the p130CAS (F15) mutant and the p130CAS RNAi demonstrate a requirement of p130CAS in axon attraction by netrin-1.

**p130CAS is involved in commissural axon projection in vivo**

Experiments discussed above have shown that p130CAS plays an important role in netrin signaling and netrin function in vitro. To determine the in vivo functional role of p130CAS, we examined the phenotype of p130CAS (F15) mutant and p130CAS RNAi on commissural axon projection in the chick embryos.

Venus YFP was introduced by electroporation together with a control vector, or with the p130CAS (F15) mutant into the neural tube of stage 12 chick embryos. Embryos were allowed to develop until stage 23 when they were killed and the lumbosacral segments of the spinal cord were isolated. The open-book preparation was made and immunostained with the anti-axonin-1 antibody, which recognized the commissural axons (Stoeckli et al., 1997). More than 90% of axons expressing Venus YFP were marked commissural axons (Fig. 8d–f), whereas p130CAS shRNA was coelectroporated with Venus YFP and p130CAS shRNA, 71.6 ± 4.0% for shRNA and wild-type group (p < 0.001 between Venus YFP and shRNA group; p < 0.0001 between vector and shRNA group; p < 0.0001 between shRNA and shRNA plus wild-type group. Student’s t-test). h, Quantification of the average distance of axons away from the floor plate: 7.2 ± 0.9 for Venus YFP; 20.9 ± 3.3 for siRNA; 7.6 ± 1.5 μm for the control vector; 44.3 ± 5.4 μm for p130CAS shRNA; 11.4 ± 2.3 for shRNA plus wild type (p < 0.01 between Venus YFP and siRNA group; p < 0.0001 between vector and shRNA group; p < 0.0001 between shRNA and shRNA plus wild-type group). The numbers on the top (n) indicate the number of embryos tested. Scale bar, 100 μm. Error bars are SEM.
hibited the projection of commissural axons (see Fig. 8h,i,j, for an example and Fig. 8k,l, for statistical analysis).

The effect of p130CAS (F15) was further confirmed with the p130CAS shRNA and siRNA (Fig. 9). Although the majority of the commissural axons in the spinal cord transfected with the control vector or Venus YFP reached the floor plate (Figs. 8b–d, 9a–c,m,n), only a small number of axons in p130CAS shRNA or siRNA transfected group reached the floor plate (Fig. 9d–l,m,n). Although the open-book preparation showed obvious defects of commissural axon projection in vivo, it was difficult to evaluate the axon turning. To examine whether the knock-down of p130CAS disrupts the commissural axon turning in addition to inhibiting axon extension in vivo, we cut the transverse section of chick spinal cord (stage 23) after electroporation (Fig. 10). In addition to the inhibition of axon extension, some commissural axons transfected with p130CAS shRNA or siRNA were misguided (Fig. 10c,c’), compared with control groups (Fig. 10a,a’,b,b’). Overexpression of wild-type p130CAS rescued the effects of p130CAS RNAi knock-down on commissural axon extension and turning (Figs. 9j–n, 10d,d’). The p130CAS knock-out mice are currently not available, and instead we introduced the Venus YFP construct and p130CAS shRNA into mice neural tube at E10.5 and did electroporation in vivo. The embryos were killed at stage 12.5, and the transverse sections of spinal cords were obtained. The projection of some commissural axons was decreased and misguided in RNAi group (Fig. 10g,g’) compared with the Venus YFP group (Fig. 10c,c’) and vector group (Fig. 10f,f’). These results indicate p130CAS is involved in the projection and pathfinding of commissural axons in vivo.

Discussion

Our results indicate p130CAS is an important component in the netrin signaling pathway acting between tyrosine kinases and the small GTPase, and p130CAS is essential for commissural axon guidance.

Previous studies have shown the involvement of multiple molecules in netrin signaling, but it was not clear whether they represent different pathways or whether they converge. For example, although the cytoplasmic tyrosine kinases Fyn and FAK (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004) and the small GTPases Rac1 and Cdc42 (Li et al., 2002; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005) are the most extensively studied molecules in netrin signaling, their relationship was unknown. Results shown here demonstrate that p130CAS is downstream of Fyn and FAK and upstream of Rac1 and Cdc42.

Our results show that p130CAS is expressed in the commissural axons in the embryonic spinal cord and colocalized with DCC. We have several pieces of biochemical evidence that place p130CAS in the netrin signal transduction pathway. In both primary neurons and HEK cells, netrin can stimulate tyrosine phosphorylation of p130CAS (Figs. 2, 3). Netrin also stimulates the formation of a protein–protein interaction complex with Fyn and FAK (Fig. 2f,d). Netrin stimulation of p130CAS phosphorylation requires DCC (Fig. 2d,h,i). p130CAS is downstream of FAK and Src family kinases, because the inhibition of FAK and Src kinases decreases netrin-induced p130CAS phosphorylation (Fig. 3a,b), which is consistent with the finding that the same domains in DCC required for FAK phosphorylation (Li et al., 2004; Ren et al., 2004) are required for netrin-induced phosphorylation of p130CAS (Fig. 2h,i). Unlike the relationship between the Src kinases and FAK, which are mutually dependent on each other (Li et al., 2004; Liu et al., 2004; Ren et al., 2004), p130CAS is not required for netrin-induced phosphorylation of FAK and Fyn, because dominant-negative mutants of p130CAS could not inhibit the phosphorylation of FAK and Fyn (Fig. 3c–e), and netrin-

![Figure 10. Wild-type p130CAS rescue of commissural defects caused by p130CAS RNAi. The chick neural tube was electroporated with Venus YFP only (a, d’), Venus YFP plus shRNA vector (b, b’), Venus YFP plus shRNA (e, e’), or Venus YFP plus shRNA plus wild-type p130CAS (d, d’). a’–d’ are the monochrome images of a–d. p130CAS shRNA not only inhibited the commissural axon extension but also caused aberrant pathfinding (c, c’). Overexpression of wild-type p130CAS rescued the defect of p130CAS shRNA on commissural axon projection and turning (d, d’). The effect of p130CAS shRNA on the spinal cord commissural axon projection was also observed in embryonic mice. E10.5 mouse neural tube was electroporated with Venus YFP only (e, e’), Venus YFP plus shRNA vector (f, f’), or Venus YFP plus shRNA (g, g’). e’, f’, and g’ are images of e, f, and g. The arrow shows a misguiding axon, and the arrowhead indicates the short commissural axon. Scale bar, 100 μm.](image-url)
induced phosphorylation of FAK could not be blocked by eliminating p130CAS (Fig. 3f). Therefore, p130CAS is clearly downstream of FAK and Src kinases in the netrin pathway.

The Rho family of small GTPases plays important roles in growth cone motility and axon guidance. Netrin can stimulate both Rac1 and Cdc42, and netrin function requires both of them (Li et al., 2002; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). Our results show that inhibition of p130CAS blocks netrin-1-induced activation of Rac1 and Cdc42 (Fig. 4), indicating that p130CAS acts upstream of Rac1 and Cdc42 in netrin-1 signaling. Because p130CAS cannot directly activate Rac1 and Cdc42, it will be interesting to identify the molecule that links p130CAS to Rac1 and Cdc42. Similarly, there are no studies linking any of the other components previously implicated in netrin signaling such as cyclic nucleotides, PLC, PI3K, MAP kinases, or calcium.

The functional roles of p130CAS were studied by both in vitro and in vivo experiments. Netrin-1 can stimulate the growth of neurites from cortical neurons. The p130CAS RNAi inhibited neurite outgrowth induced by netrin-1 (Fig. 5). Using the turning assay, we also show that both the dominant-negative p130CAS mutant F15 and p130CAS RNAi inhibited axon attraction by netrin-1 (Figs. 6, 7). These results indicate that p130CAS is required for netrin function. In vivo studies with chick and mouse spinal cord show that F15 and p130CAS RNAi cause failure of commissural axons to reach the floor plate (Figs. 8, 9). When either netrin or DCC is defective in mice (Serafini et al., 1996; Fazeli et al., 1997), the projection of commissural axons is also defective. However, other cues have also been implicated in commissural axon guidance. Bone morphogenetic proteins contribute as a repellent to the initial guidance of commissural axons in a ventral direction (Augsburger et al., 1999). Sonic hedgehog has been thought to be an attractant made in the floor plate for the commissural axons. Our result from the open-book turning assay suggests that p130CAS is not involved in mediating the repulsive response of commissural axons to the roof plate (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). However, it remains to be determined whether p130CAS is involved in the downstream signaling pathway(s) of other guidance cues.

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