Brief Communications

Draxin Inhibits Axonal Outgrowth through the Netrin Receptor DCC

Giasuddin Ahmed,1,2,3 Yohei Shinmyo,1,2 Kunimasa Ohta,1 Shahidul M. Islam,1,2 Mahmud Hossain,1,3 Iftekhar Bin Nasir,1,2,3 M. Asrafuzzaman Riyadh,1,2,3 Yuhong Su,1,2,3 Sanbing Zhang,1,2,3 Marc Tessier-Lavigne,4 and Hideaki Tanaka1,2,3

1Division of Developmental Neurobiology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan, 221st Century Center of Excellence Cell Fate Regulation Research and Education Unit, and 3Global Center of Excellence Cell Fate Regulation Research and Education Unit, Kumamoto University, Kumamoto 860-0811, Japan, and 4Laboratory of Brain Development and Repair, Rockefeller University, New York, New York 10065

Draxin, a recently identified axon guidance protein, is essential for the formation of forebrain commissures, and can mediate repulsion of netrin-stimulated spinal commissural axons. Here, we report that draxin binds multiple netrin receptors: DCC (deleted in colorectal cancer), Neogenin, UNC5s (H1, H2, H3), and DSCAM (Down’s syndrome cell adhesion molecule). Since draxin and Dcc knockouts showed similar phenotype in forebrain commissures formation, we show here the functional importance of draxin/DCC interaction. Draxin interacts with subnanomolar affinity to the netrin receptor DCC, in a region of DCC distinct from its netrin-binding domain. In vitro, neurite outgrowth from cortical and olfactory bulb explants of Dcc knock-out mice is significantly less inhibited by draxin, when compared with neurites from explants of wild-type mice. Furthermore, in comparison with wild-type mice, the growth cone collapse in response to draxin is largely abolished in Dcc-deficient cortical neurons. In vivo, double heteros of draxin/Dcc mice show markedly higher frequency of complete agenesis of corpus callosum than either of the single hetero. These results identify DCC as a convergent receptor for netrin and draxin in axon growth and guidance.

Introduction

The function of the nervous system depends on the establishment of precise and intricate neuronal connections. The combinatorial effects of attractive and repulsive guidance cues, present in the extracellular environment, direct axons to grow toward their intermediate targets and thus play a crucial role in forming proper connections between neurons. Four conserved families of axon guidance cues, the netrin, semaphorin, ephrin, and Slit proteins, mediate their guidance effects via receptors of the DCC (deleted in colorectal cancer) or UNC5, neuropilin/plexin, Eph, and Robo families, respectively. Although these proteins, as well as morphogens such as Shh (sonic hedgehog), Wnts, and BMPs, have been found to mediate their guidance effects via receptors of the DCC (deleted in colorectal cancer) or UNC5, neuropilin/plexin, Eph, and Robo families, respectively. Although these proteins, as well as morphogens such as Shh (sonic hedgehog), Wnts, and BMPs, have been found to regulate a wide variety of guidance decisions (for review, see Tessier-Lavigne and Goodman, 1996; Dickson, 2002; Zou and Lyuksyutova, 2007), it is expected that others await identification and will help address the immense complexity of the nervous system.

We recently reported the discovery of a novel axon guidance molecule, draxin, which shares no sequence homology with other known guidance molecules (Islam et al., 2009). In vitro, draxin can repel spinal commissural axons whose outgrowth is stimulated by netrin-1; in vivo, genetic deletion of draxin results in mild guidance defects of those axons and a dramatic loss of all forebrain commissures (Islam et al., 2009). Identification of the draxin receptor is important in elucidating the precise mechanism through which it functions. Here, we report that draxin binds multiple netrin-1 receptors and that its high-affinity interaction with DCC is required for draxin’s inhibitory effect on the cortical and olfactory bulb (OB) neurite outgrowth. The frequency of severity in corpus callosum (CC) malformation in draxin/Dcc double-hetero mice is more obvious than that in either of the single-hetero mice. This study identifies DCC as a functional receptor or receptor component for draxin.

Materials and Methods

Plasmids. Rat Dcc (Keino-Masu et al., 1996) was used in draxin binding experiments. Full-length CDNAs encoding rat Ncam, human Neogenin-VSVG, UNC5H1-myc, UNC5H2-HA, UNC5H3-myc, mouse Dcam...
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nitrophenylphosphate was used to determine empirically draxin-AP and control-AP concentrations.

Cell overlay assay and other binding experiments were performed as previously described (Keino-Masu et al., 1996; Islam et al., 2009). In the case of draxin binding in the presence of netrin-1, Dcc-transfected 293 cells were first incubated with 12 nM of netrin-1-his protein (R&D Systems) for 30 min followed by the incubation of draxin-AP-conditioned medium. Saturation binding curves and Scatchard analyses of draxin-AP binding to DCC were performed, and the dissociation constant ($K_d$) value was determined as previously described (Flanagan and Cheng, 2000).

**Pull-down assay.** Dcc-transfected cells were washed with HBSS buffer [HBSS containing 0.5 mg/ml bovine serum albumin, 0.1% (w/v) sodium azide, and 20 mm HEPES] and incubated with 1 nM draxin-AP-conditioned medium for 2 h at room temperature with occasional gentle stirring. Later, cells were lysed with lysis buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 0.1% Triton X-100) supplemented with protease inhibitors (1 mM PMSF, 10 g/ml aprotinin, 10 g/ml leupeptin). As a negative control, cells lysates from vector-transfected cell, which were incubated with draxin-AP, and Dcc-transfected cells, which were not incubated with draxin-AP, were taken. Lysates were incubated with anti-chick draxin polyclonal antibody (pAb) (1 g/ml) in immunoprecipitation (IP) buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1.5 mM CaCl2, 1.5 mM MgCl2, 0.1% Triton X-100, 0.1% CHAPS, 5% glycerol, and 0.1% BSA) for 12 h at 4°C, and complexes were retained and washed extensively on protein G-Sepharose beads (GE Healthcare). Immunoprecipitated DCC protein was separated by 7.5% SDS-PAGE and detected by Western blot using anti-DCC antibody.

**Concentrated draxin-AP- and DCC-ecto-Myc-His fusion protein-conditioned medium** was mixed together and incubated with anti-chick draxin pAb (1 g/ml) in IP buffer at 4°C for 12 h and then detected by Western blot using mouse monoclonal anti-DCC (Calbiochem). For pull down of draxin-AP by DCC-ecto-Myc-His protein, ProBond resin (Invitrogen) was used. As a negative control, draxin-AP was mixed with BSA. For the pull-down assay of draxin-AP by Robo1-ecto-Fc, protein G-Sepharose (GE Healthcare) was used.

Brains from E17 mouse embryos were dissected and lysed in lysis buffer supplemented with protease inhibitors, as described above. The mixtures were kept on ice for 30 min and centrifuged at 15,000 rpm at 4°C for 20 min. Membrane fractions were collected, and lysates were incubated with concentrated draxin-AP-conditioned medium and anti-chick draxin mAb for 12 h at 4°C. Immunoprecipitates were recovered and analyzed as described above.

**Explant cultures.** Explants from E17.5 OB and cortex of both wild-type (WT) and Dcc knock-out (KO) littermate mice were dissected as described previously (Shu and Richards, 2001) and cultured in collagen gels for 48 h in the presence of either draxin-AP or control-AP-conditioned medium (100 nM) mixed with Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), glutaMAX-I, and penicillin/streptomycin (Invitrogen). After culture, explants were stained with anti-neuron-specific β-tubulin (Tuj1) antibody (R&D Systems) in the collagen gels. Photographs were taken using a microscope (Keyence, BioRevo), and the longest neurite length per explant was measured using ImageJ software. Averages were determined from counted maximal lengths of all explants.

Figure 1. Draxin binds netrin-1 receptors. The 293 cells were transiently transfected with receptor cDNAs: A–D, Dcc; E, F, UNC5H1; G, H, UNC5H2; I, J, UNC5H3; K, L, Dscam; M, N, Nscam; O, P, Neogenin. After 48 h of post-transfection, cells were treated either with netrin-1 protein (A, B) or with draxin-AP-conditioned medium (C–L, N, P) and double immunostaining was performed using antibodies against respective receptors (A, C, E, G, I, K) and against bound netrin-1 (B) or draxin (D, F, H, J, L). Bound draxin and receptor expression were merged in a manner similar to netrin-1 binding (B) and DCC expression (A). In cases of NCAM-draxin (M, N) and Neogenin-draxin (O, P) binding assay, staining of NCAM or Neogenin protein and bound draxin were done separately, since all anti-NCAM, anti-Neogenin, and anti-draxin antibodies were rabbit polyclonal antibodies. A similar extent of draxin binding (P) and Neogenin expression (G) was observed, while draxin did not bind to the NCAM-transfected cells (H). Scale bars: A–D, M, N, 100 µm; E–L, O, P, 50 µm.

(Down’s syndrome cell adhesion molecule)-myc, and extracellular domain of rat Robo1-Fc were a kind gift from Drs. K. Itoh (Tokushima Bunri University, Tokushima, Japan), E. Fearon (University of Michigan, Ann Arbor, MI), P. Mellen (CNRS, Lyon, France), E. Stein (Yale University, New Haven, CT), and F. Murakami (Osaka University, Osaka, Japan), respectively. The extracellular domain of rat Dcc was subcloned into pEF-Myc-His vector (Invitrogen). Different types of human DCC cDNA fragments encoding DCC-ecto-His, DCC-Fnt(1–6)-HGH-His, and DCC-IgG(1–4)HGH-His (Geisbrecht et al., 2003) were a generous gift from Dr. D. J. Leahy (The Johns Hopkins University, Baltimore, MD).

**Antibodies.** Mouse monoclonal and rabbit polyclonal anti-chick draxin antibodies (Islam et al., 2009) were used for Western blot and immunoprecipitations. For the detection of netrin-1-His, NCAM, DCC, UNC5 (H1, H3), UNC5H2, Robo-ecto-Fc, fragmented DCC-HGH, and Neogenin, rabbit polyclonal anti-His (Santa Cruz Biotechnology), rabbit polyclonal anti-chicken NCAM (in house), mouse monoclonal anti-DC (Calbiochem), anti-myc (9E10, DS9H), anti-HA (Sigma), rabbit polyclonal anti-human Fc (Sigma), anti-HGH, anti-Neogenin, and goat polyclonal anti-DCC (Santa Cruz Biotechnology) were used. To analyze the forebrain commissure phenotype and the expression of DCC proteins, cryostat sections (16 µm thick) were subjected to immunofluorescence analysis (Islam et al., 2009) with antibodies: rat anti-L1 (Millipore Bioscience Research Reagents) and goat anti-human DCC (Santa Cruz Biotechnology).

**Binding assay and determination of dissociation constant for draxin–DCC binding.** 293T cells were transfected with cDNA encoding chick draxin-AP and empty AP tag vector (Islam et al., 2009) and an alkaline phosphatase assay (Flanagan and Cheng, 2000) using para-
Growth cone collapse and neurite outgrowth assay. Cortical neurons from mouse embryos at E16.5 were dissociated by trypsinization as described previously (Hata et al., 2006) and seeded at low density in 4-well dishes containing coverslips coated with poly-L-Lysine (100 μg/ml) and laminin (20 μg/ml).

For neurite outgrowth assay, neurons were cultured in neurobasal medium (B27, glutamax, penicillin-streptomycin) having equal amounts of either draxin-AP- or control-AP-conditioned medium for 48 h and were stained with anti-DCC.

For the growth cone-collapse experiment, neurons were seeded in a dish, as above, and cultured in neurobasal medium. After 60 h of postseeding, neurons were incubated with draxin-AP (100 nM) and control medium alone for 1 h at 37°C to induce collapse. Cultures were fixed later by 4% paraformaldehyde/10% sucrose for 1 h. After washing with PBS, growth cones were stained with phalloidin–sucrose for 1 h at 37°C to induce collapse. Cultures were fixed later by 4% paraformaldehyde/10% sucrose for 1 h. After washing with PBS, growth cones were stained with phalloidin–Alexa 568 (Invitrogen), and images were acquired on a microscope (Model Eclipse E600, Nikon) for scoring the collapse. To calculate the percentage of the collapse, the total number of growth cones was divided by the number of collapsed growth cones. Data were collected from at least two independent experiments and expressed as means ± SEM. In each independent experiment, at least 40 neurons were counted per group.

Mice. To breed homozygous Dcc mutant embryos, Dcc heterozygous mutant mice (Fazeli et al., 1997) were mated. For double-hetero mice analysis, Dcc and draxin single-hetero mice were mated. The day of the vaginal plug was designated E0.5. All animal procedures were conducted in accordance with institutional guidelines.

Results

Netrin-1 receptors bind draxin

We screened several candidate transmembrane proteins by transient expression in 293 cells and measured the binding of draxin-AP (alkaline phosphatase). Surprisingly, the netrin receptors DCC, Neogenin, UNC5s (H1, H2, H3), and DSCAM were found to be promising candidates because draxin-AP was bound to cells expressing those receptors but not to any of the other candidates tested (neuropilin1, FGFR1, EphB1, and Ryk). To examine whether receptor expression and bound draxin-AP colocalize, we assessed the Dcc-AP binding and netrin-1 receptors expression in receptor-transfected 293 cells by immunocytochemistry (Fig. 1A–P). We observed that draxin-AP binding completely overlapped with DCC, UNC5s (H1, H2, H3), and DSCAM expression (Fig. 1C–L) in a manner similar to netrin-1 binding (Fig. 1A, B), but not with the expression of NCAM (Fig. 1M, N), a member of the Ig superfamily. We also observed a similar pattern of draxin binding and Neogenin expression (Fig. 1O, P) when draxin binding was performed separately in Neogenin-transfected 293 cells. Thus, draxin binding was restricted to netrin-1 receptor-expressing cells.

Draxin interacts with DCC with high affinity, and its binding domain in DCC is different from the netrin-1 binding domain

Among the netrin-1 receptors, only Dcc KO mice phenotype similarity with draxin knockouts (Fazeli et al., 1997; Islam et al., 2009) led us to investigate the draxin/DCC interaction thoroughly. To test whether draxin interacts directly with DCC, first we performed immunoprecipitation assays. DCC was retained in the complex precipitated by draxin-AP, as detected by immunoblot using an anti-DCC antibody (Fig. 2A). To determine whether the interaction between DCC and draxin was direct, we performed IP assays using draxin-AP and the soluble DCC ectodomain. DCC-ectodomain was specifically immunoprecipitated by draxin-AP (Fig. 2B). In addition, draxin-AP was pulled down by the DCC-ectodomain (Fig. 2C). The interaction between draxin and DCC appeared specific, because a soluble version of the Robo1 ectodomain (Robo1-ecto-Fc) did not precipitate draxin-AP (Fig. 2D). Moreover, endogenous DCC from brain lysates was precipitated by draxin-AP (Fig. 2E). The binding affinity of draxin for DCC in transfected cells was determined from the binding curve, and the Kd was 970 pm (Fig. 2F), higher than that of the DCC–netrin-1 interaction in a similar assay (Keino-Masu et al., 1996). We next sought to identify the draxin-binding domain of DCC by evaluating the binding of draxin to the
DCC is required for neurite outgrowth inhibition and growth cone collapse by draxin

DCC was reported to be expressed on cortical and OB axons (Gad et al., 1997; Shu et al., 2000), and both Dcc and draxin KO mice show severe malformation of forebrain commissures (Islam et al., 2009; Fazeli et al., 1997). Also, we recently reported that draxin repelled OB axons in vitro and lateral olfactory tract (LOT) projection in draxin−/− mice was normal, though slightly defasciculated (Ahmed et al., 2010). To determine whether the interaction of draxin with DCC is functionally important, we tested the ability of Dcc-deficient cortical and OB axons to respond to draxin in vitro. Explants from E17.5 cortex and OB of Dcc KO and WT littermate mice were cultured in the presence of either draxin-AP- or control-AP-conditioned medium. Robust neurite outgrowth from both cortical and OB explants of WT mice was observed in control-AP-conditioned medium (Fig. 3B, D), and a similar degree of outgrowth was observed from explants of Dcc knock-out mice in the presence of control-AP (Fig. 3B, F). Draxin-AP significantly inhibited neurite outgrowth from cortical and OB explants of WT mice to 38 and 16%, respectively, of control levels (Fig. 3B, F). The data were obtained from two independent experiments, and growth cone collapse was assessed by counting the number of explants with intact growth cones or growth cones with significant collapse (Fig. 3D, H, I, J). These findings demonstrate that DCC is substantially required for draxin to inhibit neurite outgrowth.
Draxin not only inhibits neurite outgrowth but also induces growth cone collapse when added acutely and uniformly to growth cones in culture (Islam et al., 2009). We therefore investigated whether DCC is required for this acute response. For this assay, we used E16.5 mouse cortical neurons, which express DCC and were inhibited by draxin (Fig. 3M,N).

The specificity of the antibody was confirmed when DCC immunoreactivity was observed in the sections of WT but not of Dcc KO mice (Fig. 3K,L). We found that growth cones in this culture system had a high baseline level of collapse (∼35%), which was further increased (to ∼85%) when treated with draxin-AP (Fig. 3O,P). This increase was markedly reduced (to ∼50%) in cortical neurons derived from Dcc KO mice (Fig. 3P).

**Dcc/draxin double-hetero embryos exhibit severe defects in CC**

It was reported earlier that both Dcc and draxin single-knock-out mice showed severe defects in forebrain commissure formation and mild disruption or defasciculation of LOT projection (Fazeli et al., 1997; Islam et al., 2009; Kawasaki et al., 2006; Ahmed et al., 2010). To evaluate whether genetic interaction between Dcc and draxin is functionally important for the formation of forebrain commissures and LOT, we mated draxin and Dcc single-hetero mice. Mating of these mice generated babies with the following four different genotypes: daxin+/−;Dcc+/− (WT); daxin−/−;Dcc+/−; daxin+/−;Dcc−/−; and daxin+/−;Dcc−/−. First, we compared the forebrain commissure phenotypes in babies belonging to daxin+/−;Dcc−/−, daxin−/−;Dcc+/−, and daxin−/−;Dcc+/− mice. We observed notable variation in severity in CC defect but not in anterior and hippocampal commissure defect in single- and double-hetero mice. We classified the phenotype into two categories depending on the severity in CC defects. The CC axons in mice in the strong phenotype group failed to cross the midline and projected ventrally (Fig. 4C), while mice in the weak group showed partial misprojection near the midline (Fig. 4B). Whereas the frequencies of strong phenotypes observed in daxin and Dcc single-hetero mice were 0 and 11%, respectively, double-hetero daxin/Dcc mice showed a remarkably higher (62%) frequency of strong phenotype (Fig. 4D). Next, we checked whether LOT formed and reached normally to the telencephalon of these analyzed mice. We found that LOT formation was not disrupted and reached normally in all the single- and double-hetero mice (data not shown). This was not unexpected since LOT was normally formed in both daxin and Dcc single-knock-out mice despite mild defasciculation (Kawasaki et al., 2006; Ahmed et al., 2010). In these double-hetero mice, other guidance molecules and their receptors having redundant function to draxin might compensate the role of draxin in LOT formation. Overall, these data indicated that the aggravated CC defect in double-hetero mice could be a result of genetic interaction between daxin and Dcc genes.

**Discussion**

We have shown here that draxin binds multiple netrin-1 receptors by cell overlay assay; its interaction with DCC is especially functionally important since Dcc-lacking cortical and olfactory neurites were substantially less sensitive to draxin in vitro. Furthermore, the degree of growth cone collapse of Dcc KO cortical neurites in response to draxin was significantly lower than that of wild-type neurons. In vivo, draxin/Dcc double-hetero mice showed severe defects in CC compared with either of the single heteros. Together, these data indicate that DCC is a functionally important receptor for draxin-mediated neurite outgrowth inhibition and guidance.

Because DCC is a receptor for netrin-1, it will be of interest to determine how DCC can mediate signaling cascades for two such unrelated ligands, especially since we previously showed that spinal commissural axons can simultaneously respond to netrin-1 with outgrowth and to draxin with repulsion. The answer may lie partly in the structure of DCC, which in principle could bind different ligands through different binding domains. In fact, our data show that the draxin binding site on DCC is different from that of netrin-1. A growing body of literature indicates that the interaction of DCC with netrin-1 can lead to switching on or off of diverse intracellular processes (Ming et al., 1997; Li et al., 2004; Moore et al., 2008). There is a possibility that the downstream events that result from the DCC–draxin interaction are complementary to those triggered by DCC–netrin-1 binding. In addition, the DCC–draxin interaction might recruit other molecules as coreceptors; for example, DCC–netrin-1 can recruit UNC5 family members to mediate a repulsive action of netrin-1 (Hong et al., 1999). The probability of this notion is high since draxin also binds with UNC5s. Also, the possibility of a coreceptor that can also bind draxin is suggested by the finding that the inhibitory effect of draxin was not completely abolished by removal of DCC; this would parallel the situation with netrin, since UNC5 family members can also bind netrin and by themselves mediate repulsion, though a DCC–UNC5 coreceptor is a more potent mediator of repulsion (Keleman and Dickson, 2001).

While a conserved role for DCC in mediating netrin responses, both attractive and repulsive, has been well documented in multiple species, in Caenorhabditis elegans, the DCC homolog UNC-40 was also shown to collaborate with the Slit receptor Sax-3/Robo in mediating repulsion in response to SLT-1/Slit (Yu
et al., 2002); however, DCC family members do not appear to bind Slit proteins, and this effect appears instead to reflect a direct interaction of the receptors UNC-40/DCC with Sax-3 (Yu et al., 2002). Our results extend the principle of DCC involvement in mediating the effects of distinct ligands, but indicate that it can also involve direct binding of the ligand, as we show here for draxin. Since commissural axons can be simultaneously stimulated to extend by netrin-1 and repelled by draxin, it will be of particular interest to determine how DCC can be involved at the same time in mediating these apparently opposite functions. The identification of DCC as a receptor for draxin is an important step to further elucidation of downstream components of DCC–draxin signaling.

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