Phosphorylation of DCC by Fyn mediates Netrin-1 signaling in growth cone guidance

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Netrin-1 acts as a chemoattractant molecule to guide commissural neurons (CN) toward the floor plate by interacting with the receptor deleted in colorectal cancer (DCC). The molecular mechanisms underlying Netrin-1–DCC signaling are still poorly characterized. Here, we show that DCC is phosphorylated in vivo on tyrosine residues in response to Netrin-1 stimulation of CN and that the Src family kinase inhibitors PP2 and SU6656 block both Netrin-1–dependent phosphorylation of DCC and axon outgrowth. PP2 also blocks the reorientation of *Xenopus laevis* retinal ganglion cells that

occurs in response to Netrin-1, which suggests an essential role of the Src kinases in Netrin-1–dependent orientation. Fyn, but not Src, is able to phosphorylate the intracellular domain of DCC in vitro, and we demonstrate that Y1418 is crucial for DCC axon outgrowth function. Both DCC phosphorylation and Netrin-1–induced axon outgrowth are impaired in *Fyn*$^{-/-}$ CN and spinal cord explants. We propose that DCC is regulated by tyrosine phosphorylation and that Fyn is essential for the response of axons to Netrin-1.

Introduction

During the development of the central nervous system (CNS), axons are guided to their appropriate targets in response to extracellular cues. At the leading edge of the axons, the growth cone acts as a sensory motor structure to detect and respond to attractive and repulsive cues (Tessier-Lavigne and Goodman, 1996; Guan and Rao, 2003). These guidance cues can be either membrane-bound factors or secreted molecules, acting over short or long distances, respectively, to guide the growth of axons. They include members of the Netrin, ephrin, semaphorin, slit, receptor protein tyrosine phosphatase, and neurotrophin families of protein (Huber et al., 2003). In a more recent study, morphogens for embryonic patterning have also been implicated in axon guidance (Charron et al., 2003). Netrins belong to a small family of bifunctionally and phylogenetically conserved secreted proteins (Chisholm and Tessier-Lavigne, 1999). In the developing spinal cord, Netrin-1 attracts commissural axons toward the ventral midline but also repels other classes of neurons

(Kennedy et al., 1994; Serafini et al., 1994). The conserved transmembrane proteins belonging to the deleted in colorectal cancer (DCC) family of Netrin receptors mediate the chemoattractant effect of Netrin-1 (Keino-Masu et al., 1996; Serafini et al., 1996), but they can also participate in repulsion (Chan et al., 1996; Hong et al., 1999; Merz et al., 2001). The uncoordinated (UNC) 5 family of receptors seems to be involved exclusively in the repulsive effects mediated by Netrins, either alone or in combination with DCC (Leonardo et al., 1997).

The actin cytoskeleton plays a prominent role in the response of axons to guidance cues (Luo, 2002). Recent evidence has implicated the Rho family of small GTPases, in particular RhoA, Rac1, and Cdc42, as important signaling molecules downstream of most, if not all, guidance cue receptors (Lundquist, 2003). Indeed, the Rho family of proteins mediates a cascade of responses from the guidance cue receptors to actin remodeling (Huber et al., 2003). We and others have demonstrated that Rac1 is an important mediator of the signaling response to the Netrin-1 receptor DCC (Li et al., 2002b; Shekarabi and Kennedy, 2002) and that the adaptor protein Nck-1 couples DCC to the activation of Rac1 through a mechanism that remains to be determined (Li et al., 2002a).

Several lines of evidence also indicate that phosphotyrosine (pY) signaling is implicated in the development of the

Figure 1. **DCC is rapidly phosphorylated in vivo upon Netrin-1 stimulation in embryonic rat CN.** (A) Phase-contrast image of E13 rat CN 72 h after plating on laminin. Bar, 40 μm. (B) Immunofluorescence of CN with anti-DCC antibodies showing DCC expression in the cell bodies and along the axons. Bar, 20 μm. (C) E13 rat CN labeled with [^{32}P]orthophosphate for 2 h were either stimulated or not with Netrin-1 for 5, 10, and 30 min. Endogenous DCC was immunoprecipitated (IP) from the cell lysates. The radiolabeled proteins were subjected to SDS-PAGE and identified by autoradiography. The membrane was immunoblotted with anti-pY and anti-DCC antibodies to show the total amount of DCC. (D) Quantitative analysis of the phosphorylation level of DCC after Netrin-1 stimulation of rat CN. Fold increase in total phosphorylation of DCC was determined by densitometry ($n = 3$). Error bars represent SD. (E and F) The bands corresponding to phosphorylated DCC obtained after 5 min (E) or 30 min (F) of Netrin-1 stimulation were subjected to a phospho–amino acid analysis. (G) Quantitative analysis of DCC phosphorylation on tyrosine residues, using the method described in D ($n = 3$). Error bars represent SD.
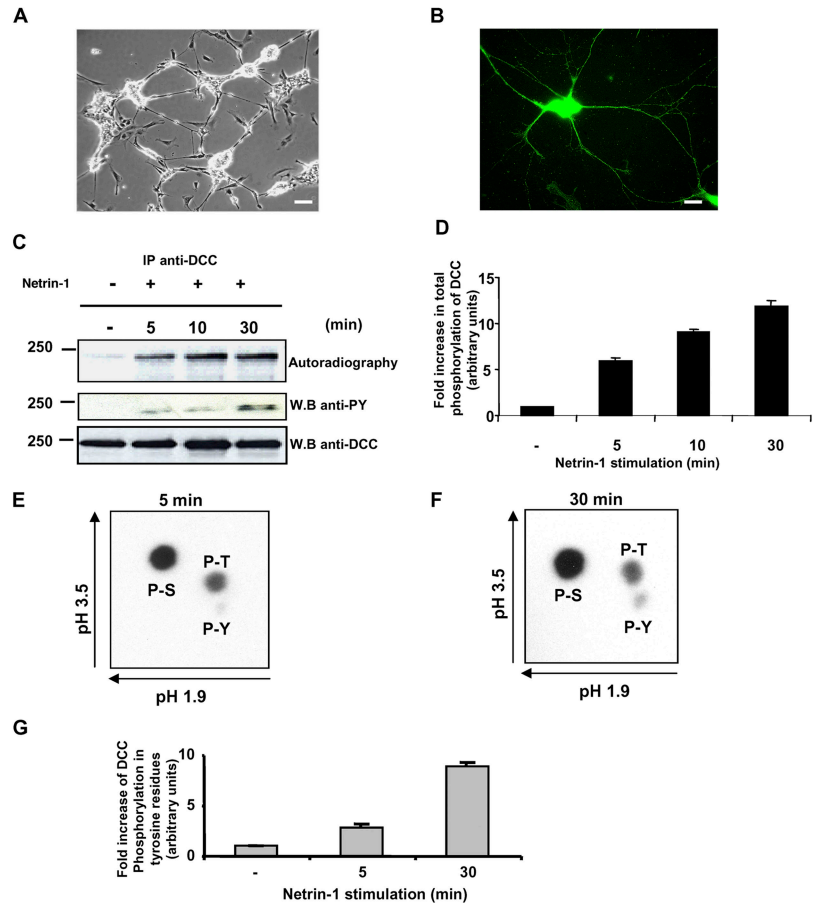
nervous system (Desai et al., 1997). Recent studies have illustrated that several guidance receptors, although lacking intrinsic kinase activity, may nonetheless serve as substrates for tyrosine kinases and could be regulated by tyrosine phosphorylation (Bashaw and Goodman, 1999; Tamagnone et al., 1999). In the case of the Netrin receptors, *Caenorhabditis elegans* UNC-5 and its mouse orthologue UNC-5H3/RCM (rostral cerebellar malformation) have been shown to be phosphorylated on tyrosine in vivo in response to Netrin-1 (Tong et al., 2001). More importantly, phosphorylation of cytoplasmic tyrosine 482 appears to be critical for UNC-5 function in vivo (Killeen et al., 2002).

To determine whether protein phosphorylation is implicated in the regulation of the Netrin-1–DCC signaling pathways, we combined the dissection and isolation of primary commissural neurons (CN) with in vivo metabolic radiolabeling techniques to examine the phosphorylation status of DCC in embryonic spinal CN. In the present paper, we provide the first evidence that Netrin-1 stimulates phosphorylation of DCC on serine, threonine, and tyrosine residues in vivo. Netrin-1–dependent phosphorylation of DCC is completely abolished in the presence of tyrosine kinase inhibitors, which suggests that tyrosine phosphorylation of DCC is a prerequisite step for DCC phosphorylation on serine and threonine residues. Furthermore, treatment of embryonic day (E) 13 rat dorsal spinal cord explants with either of the Src family kinase inhibitors PP2 or SU6656 inhibits axon outgrowth induced by

Netrin-1, which suggests that Src family kinases participate in Netrin-1 signaling. Indeed, here, we show that the intracellular domain of DCC is phosphorylated by Fyn and that phosphorylation of Y1418 is required for DCC function. Therefore, we propose that Fyn initiates Netrin-1 signaling via the phosphorylation of Y1418 of DCC.

Results

Netrin-1 induces DCC phosphorylation in vivo in embryonic spinal CN

We first examined whether endogenous DCC is phosphorylated in vivo upon Netrin-1 stimulation in dissociated CN. For this purpose, we dissected the first half of E13 rat dorsal spinal cords that contained the cell bodies of the CN (Tessier-Lavigne et al., 1988). Dissociated CN exhibit long and fasciculated axons enriched in growth cones at the tips of the axons after 72 h in culture (Fig. 1 A). As shown in Fig. 1 B, a high level of DCC expression is observed in the cell bodies and along the neurite extensions as described previously (Li et al., 2002b; Shekarabi and Kennedy, 2002). Thus, we chose this time point to examine the phosphorylation of DCC in vivo in response to Netrin-1. 72 h after plating on laminin-1–coated dishes, the CN were serum-starved and then incubated in phosphate-free medium supplemented with [^{32}P]orthophosphate for 2 h before stimulation with Netrin-1 for different periods of time. Phosphorylation of endogenous DCC is increased 5 min after stim-
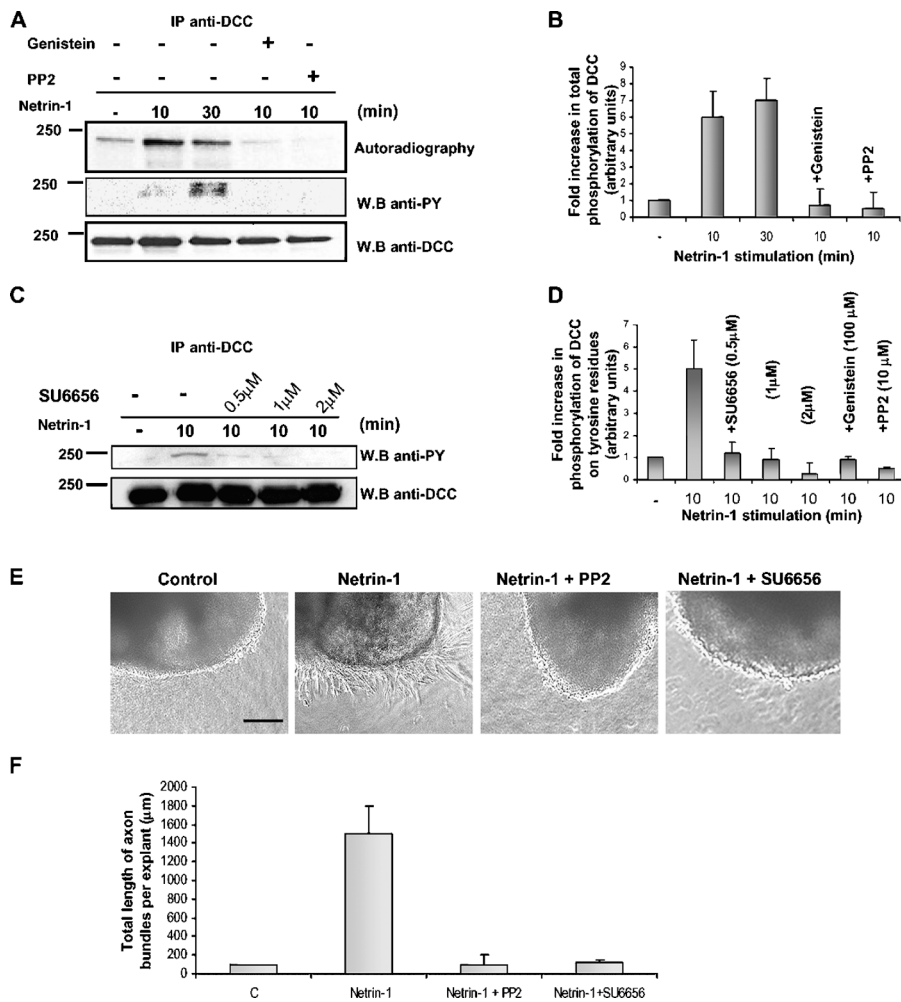
Figure 2. DCC phosphorylation on tyrosine residues is Src family kinase dependent and is critical for Netrin-1–mediated axon outgrowth. (A) Labeling of CN was or was not followed by stimulation with Netrin-1 for 10 or 30 min. The neurons were also treated or not, for 2 h, with either genistein or PP2 before a 10-min Netrin-1 stimulation. (C) Nonlabeled rat CN were also treated for 2 h with different concentrations of SU6656 before a 10-min Netrin-1 stimulation. (A and C) DCC was immunoprecipitated (IP) from the cell lysates and the products were analyzed by SDS-PAGE and autoradiography. The membrane was immunoblotted with anti-pY and anti-DCC antibodies to analyze the total amount of DCC. (B) Quantitative analysis of the total DCC phosphorylation after genistein or PP2 treatments (n = 4). (D) Quantitative analysis of DCC phosphorylation on tyrosine residues after genistein, PP2, or SU6656 treatments. (B and D) Error bars represent SD. (E) E13 rat dorsal spinal cord explants were cultured for 36 h, alone (control), in the presence of Netrin-1, or with both Netrin-1 and either PP2 or SU6656. Bar, 100 μm. (F) Quantification of the total length of axon bundles per explant in micrometers. Error bars represent SD.

ulation with Netrin-1, and a 12-fold increase in the level of DCC phosphorylation is observed after 30 min of stimulation (Fig. 1, C and D). To assess the content of phosphorylated residues on DCC upon Netrin-1 stimulation, we performed a phospho–amino acid analysis of immunoprecipitated DCC after 5 and 30 min of incubation with Netrin-1. Interestingly, DCC is mainly phosphorylated, in vivo, on serine and threonine residues, and to a lesser extent on tyrosine residues (Fig. 1, E and F). The eightfold increase in tyrosine phosphorylation of DCC after 30 min of Netrin-1 stimulation (Fig. 1, F and G) is confirmed by Western blotting using anti-pY antibodies on immunoprecipitated DCC from embryonic commissural neuron lysates (Fig. 1 C). Therefore, Netrin-1 induces DCC phosphorylation in vivo on serine, threonine, and tyrosine residues in embryonic CN.

Netrin-1–induced phosphorylation of DCC requires Src family tyrosine kinase activity

Because the Src family kinases, particularly the Src and Fyn members, play pivotal roles in neuronal signaling cascades (Sperber et al., 2001), we examined the implication of the Src family in the phosphorylation of DCC. In vivo, [^{32}P]orthophosphate-labeled CN were left untreated or treated with either the

wide-spectrum tyrosine kinase inhibitor genistein (Akiyama et al., 1987) or one of the two different Src family kinase specific inhibitors PP2 (Hanke et al., 1996) and SU6656 (Blake et al., 2000) before a 10-min stimulation with Netrin-1. By Western blotting using anti-pY antibodies, we show that tyrosine phosphorylation of DCC is completely inhibited when the cells have been treated with genistein (Fig. 2, A and B). PP2 as well as SU6656 treatments of the CN also lead to the inhibition of DCC tyrosine phosphorylation, which indicates that the Src kinases are implicated in this phosphorylation event (Fig. 2, A, C, and D). Interestingly, immunoprecipitation of ^{32}P-radiolabeled DCC reveals that both genistein and PP2 significantly decreased the total phosphorylation of DCC in response to Netrin-1 (Fig. 2, A and B). Because serine residues account for most of the phosphorylation sites in DCC (Fig. 1, E and F), these data suggest that Src family kinase activity is critical for initiation of the molecular events leading to Netrin-1–dependent phosphorylation of DCC.

Src family kinases are required for axon outgrowth and attraction induced by Netrin-1

To address the physiological significance of phosphorylation of DCC, we examined the question of whether Src fam-
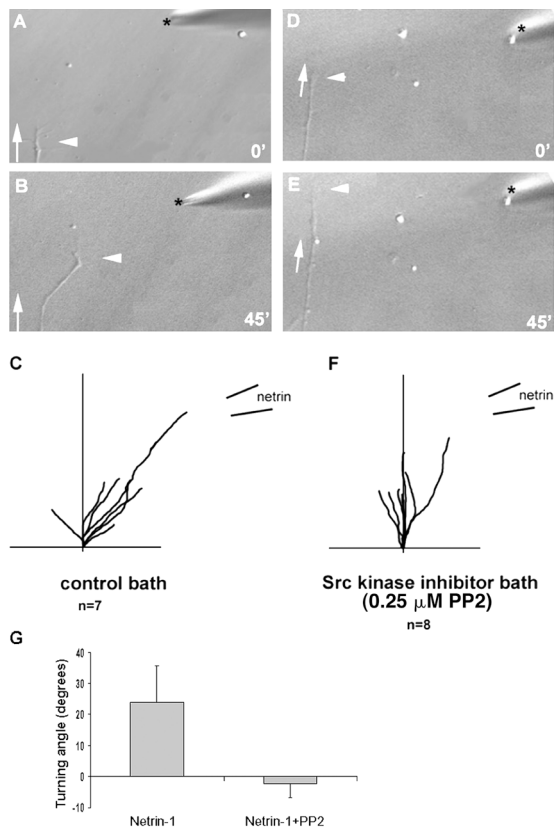
Figure 3. **Inhibition of Src family kinases abolishes Netrin-1–induced turning response of RGC growth cones.** (A, B, D, and E) *X. laevis* RGC growth cones extending from a 24-h stage 24 explant culture. Pulsatile ejection of Netrin-1 from a pipette (*) with a 0.5–1.0-μm opening was used to set up a concentration gradient next to an extending RGC growth cone (arrowheads). (A and D) Trajectories of growth cones before applying the pipette solution (arrows). (B and E) Growth cones (arrows) 45 min after continuous exposure to the Netrin-1 concentration gradient. The growth cone in the control bath (A and B) turned toward the Netrin-1 source, whereas the growth cone bathed in 0.25 μM of Src kinase inhibitor PP2 (D and E) ignored the Netrin-1 source. (C and F) Superimposed neurite trajectories of growth cones exposed to Netrin-1 source in a control medium (C) and growth cones exposed to Netrin-1 in the presence of PP2 (F). (G) Representation of the mean turning angles of the growth cones in C and F ($P < 0.05$; unpaired two-tailed *t* test). Error bars represent SD.

ily kinases are involved in mediating the axon outgrowth–promoting effects of Netrin-1. Explants of E13 rat dorsal spinal cord cultured in a three-dimensional collagen gel in the presence of 160 ng/ml of Netrin-1 show maximal commissural axon outgrowth (Fig. 2, E and F), as reported previously (Serafini et al., 1996). When PP2 or SU6656 were added in the presence of Netrin-1 to the explants, the increase in axon outgrowth was completely abolished, similar to the results in the control (Fig. 2, E and F). These results demonstrate that inhibition of the Src kinases interferes with the effect of Netrin-1 to mediate commissural axon outgrowth, suggesting a critical role for the Src kinases in Netrin-1–DCC signaling. To determine whether Src kinases are required for Netrin-1 to mediate axon attraction, we used the in vitro turning assay in which *Xenopus laevis* retinal ganglion cells (RGC) turn toward a source of Netrin-1 (de la Torre et al., 1997). As shown in Fig. 3 B, a retinal growth

cone extending from a 24-h, stage 24 explant culture turned toward the source of Netrin-1 in the control bath. In contrast, the retinal growth cone ignored the source of Netrin-1 in the presence of PP2 (Fig. 3 E). A trace of the paths taken by all growth cones is represented in Fig. 3 (C and F). The turning angles taken by the retinal growth cones were quantified and are shown in Fig. 3 G. These results reveal the implication of Src family kinases in the turning response of growth cones to Netrin-1.

DCC is phosphorylated in vitro by Fyn tyrosine kinase

DCC comprises a large extracellular domain of four immunoglobulin repeats followed by six fibronectin type III repeats, a single transmembrane domain, and a cytoplasmic tail with three conserved motifs (P1, P2, and P3) (Grunwald and Klein, 2002; Fig. 4 A). The P3 region is involved in the ligand-gated multimerization of DCC that is required to mediate the Netrin-1–induced attraction response (Stein and Tessier-Lavigne, 2001). The P1 region interacts with the intracellular domain of the UNC-5 receptor family, inducing the heterodimerization between DCC and UNC-5 receptors that has been shown to be involved in some, but not all, repulsive events induced by Netrin-1 (Merz et al., 2001). The cytoplasmic tail of DCC contains four tyrosine residues highly conserved across rat, mouse, human, and *X. laevis* species (Fig. 4 B). Only tyrosine 1272 is conserved in Frazzled, the *Drosophila melanogaster* orthologue of mammalian DCC, and only Y1418 is conserved in neogenin, a member of the DCC family of proteins. Surprisingly, none of the four tyrosine residues is conserved in UNC-40, the *C. elegans* orthologue of DCC. The four tyrosine residues in the intracellular domain of the rat DCC are located within the limits of the P1, P2, and P3 regions, which suggests that they may play a critical role in DCC function (Fig. 4 A). Interestingly, only the motif pYEQD containing Y1418 is a likely phosphorylation target site of Src tyrosine kinases, as it resembles the known consensus sequence pYEEI for Src kinases (Songyang et al., 1993). Thus, we examined whether purified Src or Fyn directly phosphorylates in vitro the cytoplasmic domain of DCC (DCC-C). Truncated DCC lacking the majority of the extracellular domain was expressed as a GST fusion protein and purified as described in Materials and methods. As shown in Fig. 4 C, purified Fyn, but not Src, phosphorylates DCC-C after a 10-min incubation. Using a small range of DCC-C concentrations, we roughly estimated a Km value of 70 nM, which suggests that DCC is a good substrate for Fyn. To determine which tyrosine residues are phosphorylated by Fyn, each tyrosine of the intracellular domain of DCC was substituted with a phenylalanine residue. Three DCC mutant proteins containing either the Y1261F, Y1272F, or Y1361F amino acid substitutions are phosphorylated by Fyn at a similar level as DCC-C (Fig. 4, C and D; and not depicted). However, phosphorylation of DCC-C-Y1418F by Fyn is significantly reduced compared with that of DCC-C (Fig. 4, C and D), which indicates that this Y1418 is a phosphorylation target site of Fyn in vitro.
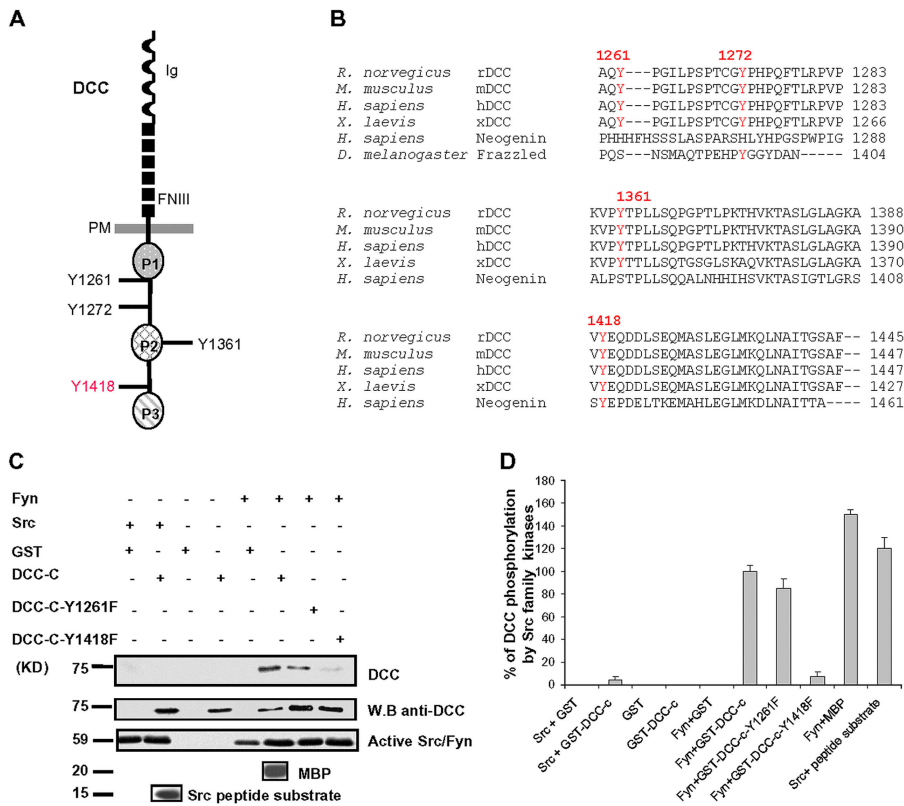
Figure 4. **The intracellular domain of DCC is phosphorylated in vitro by Fyn tyrosine kinase.** (A) The extracellular domain of DCC is characterized by four amino-terminal Ig-like domains and six fibronectin type III (FNIII) repeats, the transmembrane domain, and the cytodomain, which contains three regions according to the rat amino acid sequence: P1, P2, and P3. Protein mutants of rat DCC were created by substituting each tyrosine in the cytodomain at 1261, 1272, 1361, and 1418 positions into a phenylalanine residue. (B) Amino acid sequence alignment of DCC proteins from different species and human neogenin. The tyrosine residues corresponding to the rat amino acid sequence of DCC are shown in red. (C) 20 μg of GST fusion proteins containing the cytodomains of the wild-type DCC (DCC-C) and the DCC-C-Y1261F and DCC-C-Y1418F mutants was incubated without or with the active Fyn or Src kinases in the presence of γ-[^{32}P]ATP. As positive controls, Fyn was incubated with 5 μg MBP, and Src with 5 μg of a specific peptide substrate. The products were analyzed by SDS-PAGE followed by autoradiography, and the total amount of GST fusion proteins was determined by Western blot (W.B) with anti-DCC antibodies. Autophosphorylated bands of Fyn and Src are also shown as positive controls of their autoactivation. (D) Quantitative analysis of the percent DCC phosphorylated by Src kinases in vitro. 100% corresponds to the phosphorylation of wild-type DCC-C protein (n = 3). Error bars represent SD.

Fyn tyrosine kinase, but not Src, regulates the phosphorylation of DCC in N1E-115 neuroblastoma cells

To determine which tyrosine residues of DCC are phosphorylated in vivo, we investigated the phosphorylation status of the various DCC mutant proteins expressed in N1E-115 mouse neuroblastoma cells. We have shown previously that N1E-115 cells constitutively produce Netrin-1 but do not express DCC (Li et al., 2002b). In the presence of serum, N1E-115 cells are round and show lamellipodia formation and multiple filopodia, but they do not extend neurites. The expression of DCC in these cells induces neurite extension (Li et al., 2002b). As shown in Fig. 5 A, DCC is phosphorylated on tyrosine residues in N1E-115 cells, which is consistent with the tyrosine phosphorylation of DCC observed in rat primary CN. To confirm that phosphorylation of DCC is regulated by Fyn tyrosine kinase in N1E-115 cells, we coexpressed constitutively active (CA) Fyn or dominant negative (DN) Fyn with DCC in N1E-115 cells (Fig. 5 B). Indeed, Fyn-CA shows a 30-fold increase in the level of tyrosine phosphorylation of DCC, whereas Fyn-DN inhibits the basal level of tyrosine phosphorylation of DCC, similar to the results observed when cells are treated with PP2 or SU6656 (Fig. 5, A–D). In comparison, the expression of Src-CA in N1E-115 cells did not affect the basal level of DCC phosphorylation (Fig. 5, C and D).

To further characterize the tyrosine residue(s) phosphorylated by Fyn in vivo, we coexpressed the DCC tyrosine mutants Y1261F, Y12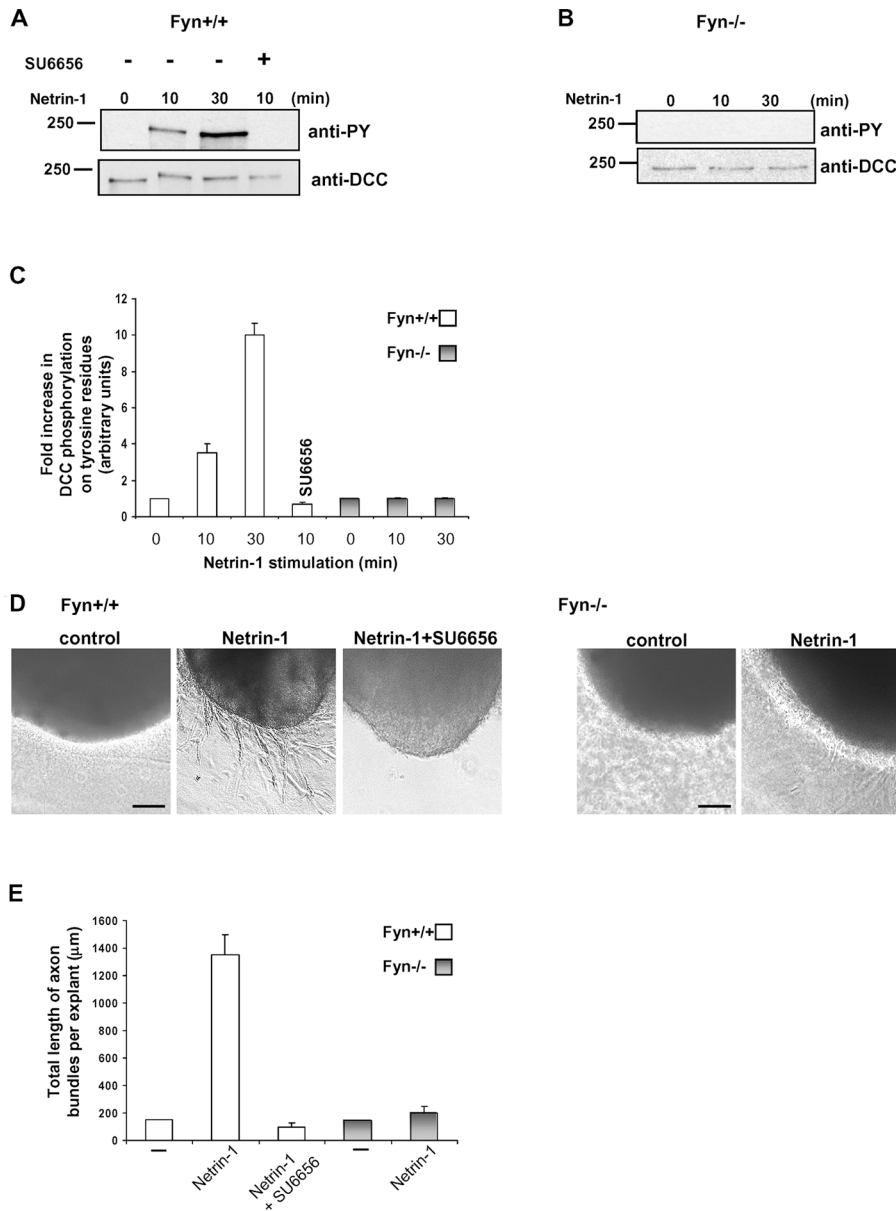72F, Y1361F, and Y1418F with Fyn-CA in N1E-115 cells. As shown in Fig. 5 (B and D), the tyrosine phosphorylation status of the DCC mutant proteins Y1272F and Y1361F is moderately decreased compared with that of wild-type DCC. The level of tyrosine phosphorylation of DCC mutant proteins containing Y1261F or Y1418F, however, shows a twofold decrease compared with that of the wild-type protein. Consistent with the data obtained in the in vitro kinase assay, these results show that DCC is phosphorylated by Fyn, but Src, in N1E-115 cells, and that tyrosines 1261 and 1418 are the major phosphorylation sites of Fyn in vivo.

Fyn tyrosine kinase activity, but not Src, is required to mediate neurite outgrowth induced by DCC

To further demonstrate the importance of the tyrosine kinase activity of Fyn on the neurite outgrowth function of DCC, N1E-115 cells were cotransfected with DCC and either Fyn-CA or Fyn-DN. We show that the expression of Fyn-DN completely inhibits neurite extensions induced by DCC, whereas the expression of Fyn-CA leads to a small increase in cells exhibiting neurites compared with cells expressing DCC alone (Fig. 5, E and F). Likewise, treatment of N1E-115 cells expressing DCC with PP2 or SU6656 abolishes the ability of DCC to induce neurite extensions, demonstrating the essential role of Src family kinases in the neurite outgrowth function of DCC (Fig. 7, A and B). The expression of Fyn-CA by itself induces the formation of neurites in N1E-115 cells, as demonstrated previously (Suetsugu et al., 2002), but to a lesser extent than that induced by
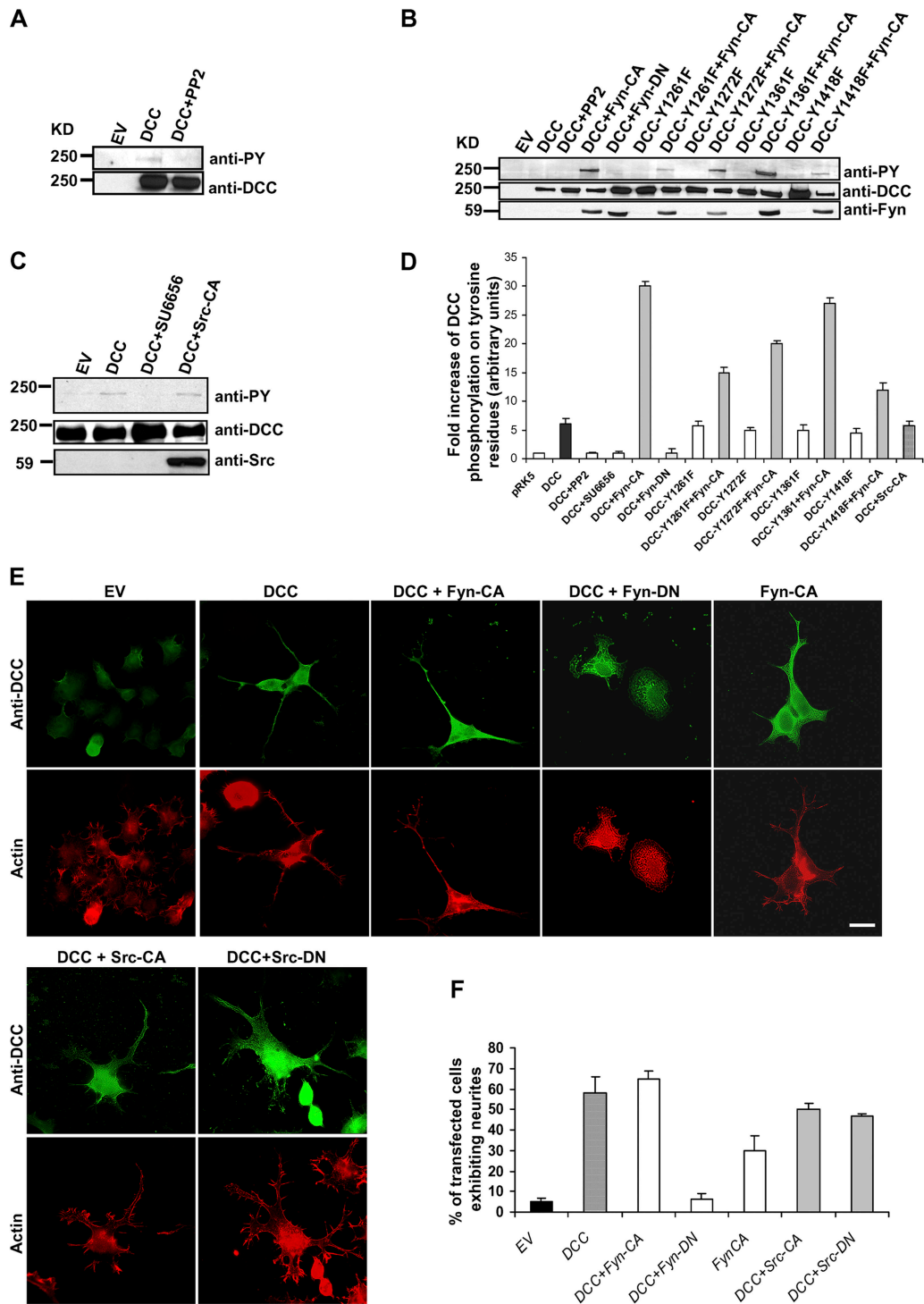
Figure 5. **Fyn tyrosine kinase regulates the phosphorylation of DCC and is critical for DCC-induced neurite outgrowth in N1E-115 cells.** (A) N1E-115 cells were transfected with empty vector (EV) or pRK5-DCC (DCC). Cells expressing DCC were treated with PP2. DCC was immunoprecipitated from the lysates, and the total amount of DCC was determined using anti-DCC antibodies. Anti-pY antibodies were used to show the level of DCC phosphorylation on tyrosines. (B) The empty vector (EV), DCC, DCC-Y1261F, DCC-Y1272F, DCC-Y1361F, or DCC-Y1418F constructs were transfected either alone or together with Fyn-CA, in N1E-115 cells. Cells expressing DCC were treated with the Src kinase inhibitor PP2. DCC was also cotransfected with Fyn-DN. The expression of these proteins was analyzed by Western blot using anti-DCC and anti-Fyn antibodies, and the phosphorylation levels of these proteins were assessed using anti-pY antibodies. (C) N1E-115 cells were transfected with empty vector (EV) or with DCC, alone or with Src-CA. DCC-transfected cells were also treated with SU6656. After DCC immunoprecipitation, the phosphorylation levels of these proteins were assessed using anti-pY antibodies, and the total amounts of the expressed proteins were determined using anti-DCC and anti-Src antibodies. (D) Quantitative analysis of the tyrosine phosphorylation of DCC and DCC mutant proteins in N1E-115 cells ($n = 3$). Error bars represent SD. (E) N1E-115 cells were transfected with empty vector (EV), Fyn-CA alone, and DCC either alone or together with Fyn-CA, Fyn-DN, Src-CA, or Src-DN. The cells were costained with anti-DCC antibodies and rhodamine-conjugated phalloidin to visualize the actin filaments. Fyn or Src expression was visualized using anti-p62 antibodies (Santa Cruz Biotechnology, Inc.) (not depicted). Bar, 20 μm. (F) Quantitative analysis of the percent of transfected N1E-115 cells exhibiting neurites shown in E ($n = 3$) was performed in a blinded fashion. Error bars represent SD.

A Fyn+/+

SU6656   −   −   −   +
Netrin-1   0   10   30   10   (min)

250 — anti-PY

250 — anti-DCC

B Fyn-/-

Netrin-1   0   10   30   (min)

250 — anti-PY

250 — anti-DCC

C

Fyn+/+ ☐
Fyn-/- ▨

D Fyn+/+

control   Netrin-1   Netrin-1+SU6656

Fyn-/-

control   Netrin-1

E

Fyn+/+ ☐
Fyn-/- ▨

Figure 6. **Netrin-1–induced DCC phosphorylation on tyrosine residues and neurite outgrowth are impaired in Fyn$^{-/-}$ mice.** (A) E11.5 $Fyn^{+/+}$ mouse CN were or were not stimulated with Netrin-1 for 10 or 30 min. The neurons were also pretreated with SU6656 before a 10-min stimulation with Netrin-1. DCC was immunoprecipitated from the cell lysates and the products were analyzed by SDS-PAGE. The membrane was immunoblotted with anti-pY and anti-DCC antibodies. (B) E11.5 CN dissected from $Fyn^{-/-}$ mice were or were not stimulated for 10 or 30 min with Netrin-1. DCC immunoprecipitates were analyzed by Western blot using anti-pY and anti-DCC antibodies. (C) Quantitative analysis of the phosphorylation level of DCC on tyrosine residues after Netrin-1 stimulation in E11.5 wild-type or $Fyn^{-/-}$ CN, determined by densitometry ($n = 3$). Error bars represent SD. (D) E11.5 dorsal spinal cord explants from $Fyn^{+/+}$ or $Fyn^{-/-}$ mice were cultured for 20 h, alone (control) or in the presence of Netrin-1. $Fyn^{+/+}$ explants were also treated with both Netrin-1 and SU6656. Bar, 100 μm. (E) Quantification of the total length of axon bundles per explant in micrometers ($n = 36$). Error bars represent SD.

DCC expression (Fig. 5, E and F). In comparison, the expression of Src-CA or Src-DN in N1E-115 cells did not affect the neurite outgrowth function of DCC (Fig. 5, E and F). These results show that the kinase activity of Fyn, but not of Src, is required for the neurite outgrowth function of DCC.

Fyn tyrosine kinase activity is essential for DCC phosphorylation and Netrin-1–dependent axon outgrowth function in vivo

To confirm that Fyn tyrosine kinase is implicated in vivo in the regulation of the phosphorylation of DCC, we examined the phosphorylation level of DCC in embryonic CN from Fyn-deficient ($Fyn^{-/-}$) mice in response to Netrin-1. As shown in Fig. 6 (A and C), a 10-fold increase in DCC phosphorylation on tyrosine residues is observed after 30 min of Netrin-1 stimulation in wild-type E11.5 CN. However, no tyrosine phosphorylation band is detected when DCC is immunoprecipitated from $Fyn^{-/-}$ CN, after

30 min of Netrin-1 stimulation, or from wild-type CN pretreated with SU6656 before a 10-min Netrin-1 stimulation (Fig. 6, A–C). Next, we examined the ability of dorsal spinal cord explants from $Fyn^{-/-}$ mouse embryos to extend neurites in response to Netrin-1, in comparison with that of $Fyn^{+/+}$ explants. As shown in Fig. 6 D, explants from $Fyn^{+/+}$ mice, treated for 20 h with Netrin-1, show maximal axon outgrowth compared with the nontreated control explants. When the Src family kinase inhibitor SU6656 is added with Netrin-1 to the $Fyn^{+/+}$ explants, the axon outgrowth is completely abolished (Fig. 6, D and E), as observed with rat dorsal spinal cord explants. However, when explants from $Fyn^{-/-}$ dorsal spinal cords are cultured in the presence of Netrin-1 for 20 h, the axon outgrowth is completely abolished in up to 90% of the total number of explants examined ($n = 36$; Fig. 6, D and E). These results are consistent with the data obtained in vitro, showing the crucial role of Fyn tyrosine kinase in DCC phosphorylation and Netrin-1–induced outgrowth function in vivo.

Figure 7. **DCC phosphorylation on tyrosine 1418 is critical for neurite outgrowth in N1E-115 cells.** (A) Immunocytochemistry of N1E-115 cells expressing DCC, Y1261F-, Y1272F-, Y1361F-, or Y1418F-DCC mutant proteins. The cells were costained with anti-DCC antibodies and with rhodamine-conjugated phalloidin to stain F-actin. Cells expressing DCC were treated with either PP2 or SU6656. Bar, 20 μm. (B) Quantitative analysis of the percent transfected N1E-115 cells exhibiting neurites shown in A (n = 4). The percent transfected N1E-115 cells exhibiting neurites was determined by counting >100 expressing cells exhibiting at least one neurite per cell. A neurite was defined as a process that measured at least the length of one cell body. Error bars represent SD.
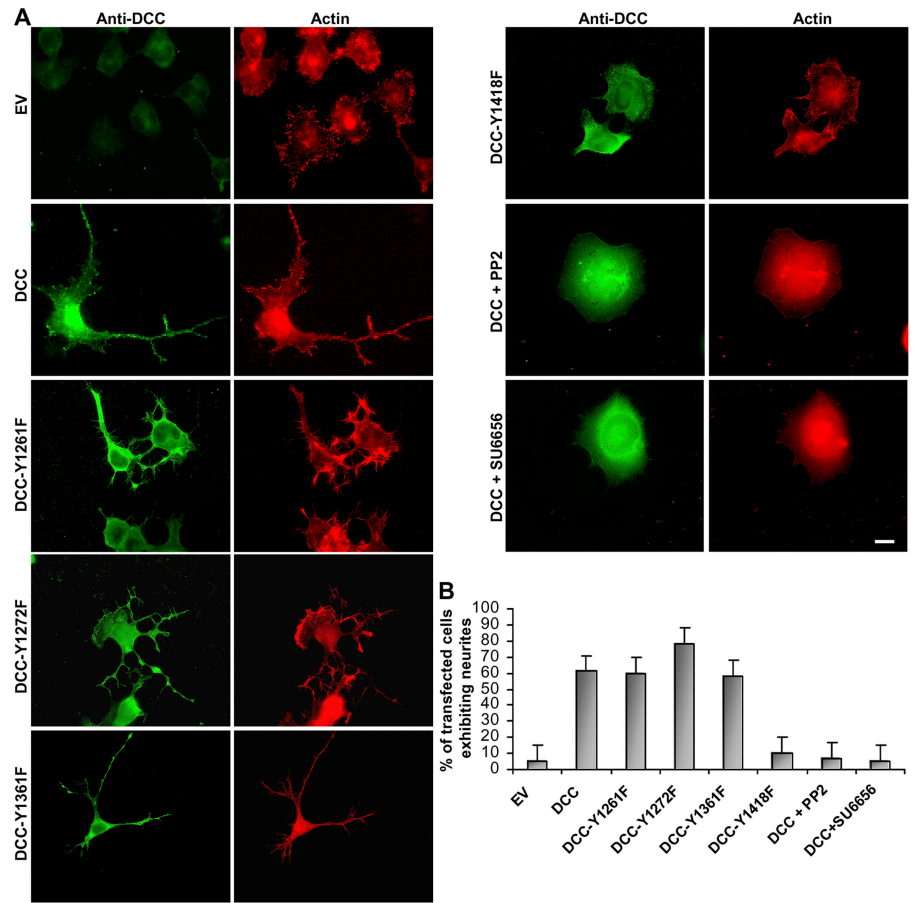
Phosphorylation of tyrosine 1418 is critical for DCC-induced neurite outgrowth in N1E-115 cells
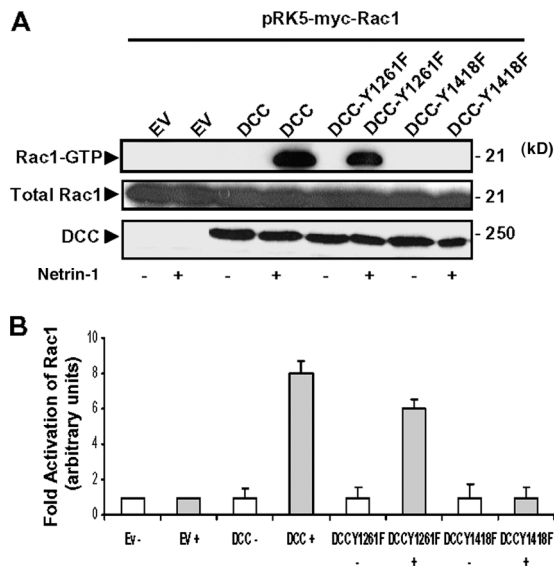
To define the importance of tyrosine phosphorylation of DCC in neurite outgrowth, we determined whether N1E-115 cells expressing the various DCC mutants can extend neurites. In the presence of 5% serum, control cells are round with some lamellipodia structures and filopodia (Fig. 7 A). In contrast, cells expressing DCC exhibit long neurite extensions with thin filopodia along the neurites (Fig. 7, A and B). The expression of Y1261F-, Y1272F-, or Y1361F-DCC mutant proteins induces neurite extensions in N1E-115 cells at levels similar to those obtained with the expression of DCC. However, expression of Y1418F mutant protein completely inhibits the ability of DCC to induce neurite outgrowth (Fig. 7, A and B). Cells expressing Y1418F mutant are flat and round with no specific actin structures, which is similar to cells treated with PP2 or SU6656. Although both Y1261 and Y1418 are phosphorylated by Fyn in N1E-115 cells, these findings indicate that only phosphorylation of Y1418 is critical for DCC to induce neurites in response to Netrin-1.

Phosphorylation of tyrosine 1418 is essential for DCC-induced activation of the small GTPase Rac1

We have previously demonstrated that expression of DCC in fibroblasts specifically activates the small GTPase Rac1 in a Netrin-1–dependent manner and that Rac1 activity is essential for DCC-induced neurite outgrowth in N1E-115 cells (Li et al., 2002b). To determine whether substitution of Y1418 by a phenylalanine residue interferes with the signaling pathways leading to Rac1 activation after Netrin-1 stimulation, we performed a pull-down assay in which GTP-loaded Rac1 was trapped by specific binding to the Cdc42/Rac interactive binding domain of p65[PAK] fused to GST (GST-PAK). DCC or the various DCC mutants were coexpressed with Myc-tagged Rac1 in COS-7 cells for 24 h and serum-starved overnight. After 5 min of Netrin-1 stimulation, protein lysates were prepared and incubated with GST-PAK, and the amount of Rac1-GTP precipitated by GST-PAK was determined by Western blot analysis. As shown in Fig. 8 (A and B), Netrin-1 stimulates an eightfold increase in the level of activated Rac1. The expression of Y1261F-, Y1272F-, or Y1361F-DCC mutant proteins shows a slight decrease in the level of activated Rac1 induced by Netrin-1 compared with that when DCC is expressed (Fig. 8, A and B; and not depicted). In contrast, activation of Rac1 by Netrin-1 is completely abolished when Y1418F mutant protein is expressed in COS-7 cells (Fig. 8, A and B). Thus, phosphorylation of Y1418 seems to be crucial for the activation of Rac1 by DCC. Together with the results obtained in N1E-115 cells, these data provide evidence for an important role of the phosphorylation of Y1418 in the signaling pathways mediated by the Netrin-1 receptor DCC, leading to neurite outgrowth.

A pRK5-myc-Rac1

EV EV DCC DCC DCC-Y1261F DCC-Y1261F DCC-Y1418F DCC-Y1418F

Rac1-GTP ▶ — 21 (kD)

Total Rac1 ▶ — 21

DCC ▶ — 250

Netrin-1   - + - + + - +

B

Figure 8. **DCC phosphorylation on tyrosine 1418 is critical for Netrin-1–induced Rac1 activation.** (A) COS-7 cells coexpressing DCC, Y1261F-, or Y1418F-DCC mutants together with Myc-Rac1 protein were or were not treated with Netrin-1 for 5 min. The GTP-loaded Rac1 were pulled down from the cell lysates using GST-Cdc42/Rac interactive binding domain-Pak1 fusion protein. The proteins from the pull-down and from the total cell lysate were analyzed by Western blot using anti-DCC antibodies and anti-Myc antibodies to detect both Rac1-GTP and the total Rac1. (B) Quantitative analysis of Rac1 activation by DCC and DCC tyrosine mutants after Netrin-1 treatment ($n = 5$). Error bars represent SD.

Discussion

We provide the first evidence that the transmembrane receptor DCC is regulated by phosphorylation in a Netrin-1–dependent manner. In dissociated CN, DCC is highly phosphorylated on serine, threonine, and tyrosine residues in response to Netrin-1. Fyn tyrosine kinase regulates DCC phosphorylation and is required to mediate axon outgrowth and attraction induced by Netrin-1. In vitro studies suggest that Y1418 in the vicinity of the P3 region of the intracellular tail of DCC is a likely phosphorylation site for Fyn, but not Src, tyrosine kinase. Phosphorylation of this tyrosine residue is essential for DCC to promote neurite outgrowth in N1E-115 cells and to mediate downstream signals leading to the activation of the small GTPase Rac1 (Fig. 9).

Biochemical and genetic studies have demonstrated the essential role of tyrosine phosphorylation in neurite outgrowth and growth cone guidance (Desai et al., 1997). In the case of Netrin signaling, tyrosine phosphorylation of the mammalian UNC-5H3 receptor has been observed in mouse brain. In addition, Netrin-1 was able to stimulate tyrosine phosphorylation of UNC-5H3 expressed in HEK-293 cells (Tong et al., 2001); however, the physiological role of UNC-5H3 tyrosine phosphorylation remains to be demonstrated. On the other hand, mutations of nine tyrosine residues in the cytoplasmic domain of *C. elegans* UNC-5 severely compromise UNC-5 function in vivo (Killeen et al., 2002). Tyrosine phosphorylation of UNC-40 in vivo has also been reported in *C. elegans*, although the role of this phosphorylation has not been determined (Tong et al., 2001). In this paper, we have demonstrated that Netrin-1
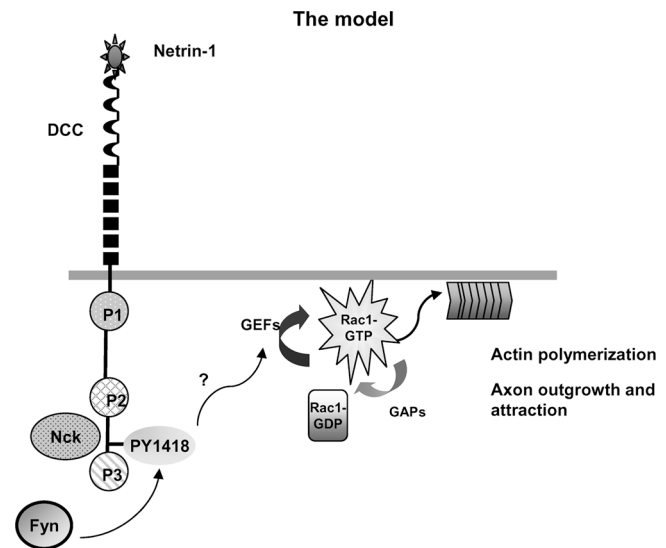
The model

Figure 9. **The model.** Netrin-1 binding to DCC induces a rapid phosphorylation of Y1418 in the vicinity of the P3 region by Fyn. This primary event might initiate directly or indirectly the activation of a specific unknown guanine nucleotide exchange factor (GEF) that activates Rac1, leading subsequently to actin assembly at the plasma membrane, axon outgrowth, and attraction.

stimulates the in vivo tyrosine phosphorylation of DCC in metabolically radiolabeled CN dissociated from the developing spinal cord. Interestingly, we have also observed that DCC is highly phosphorylated on Ser/Thr residues as well. The role of Ser/Thr phosphorylation of DCC is still unknown and will require further investigation. The interaction of DCC with the mitogen-activated protein kinases ERK1/2 and MEK1/2 and their contribution to Netrin signaling in axon outgrowth and guidance (Forcet et al., 2002) suggest that ERK1/2 and MEK1/2 are interesting candidates to investigate as potential Ser/Thr kinases for DCC.

When DCC phosphorylation is completely inhibited by the Src family kinase inhibitor PP2, Netrin-1 is no longer able to induce commissural axon outgrowth or to reorient retinal growth cones. These findings suggest that phosphorylation of DCC is essential to mediate the intracellular neuronal responses leading to axon outgrowth and attraction. Moreover, they show that Src family kinases are crucial for Netrin-1–mediated axon outgrowth and guidance. This is consistent with several studies that have implicated Src and Fyn tyrosine kinases as pivotal players in CNS development (Sperber et al., 2001). Indeed, Src, Fyn, Yes, and Lyn tyrosine kinases are expressed in the developing vertebrate CNS, where they are enriched in growth cone membrane fractions, which suggests that they may play a role in neurite outgrowth and guidance (Maness et al., 1988; Bixby and Jhbvala, 1993). However, studies from the single knockout mutants of the tyrosine kinases Src, Fyn, Yes, and Lyn revealed that only $Fyn^{-/-}$ mice display any obvious neuronal defects and reduced tyrosine phosphorylation of proteins in the brain (Grant et al., 1992). Interestingly, the inhibition of Src family kinase activity decreases the total phosphorylation of DCC below the basal level, which suggests that blocking Src family kinase activities also impairs DCC

phosphorylation on Ser/Thr residues. One possibility is that phosphorylation of specific tyrosine residues is a prerequisite step for facilitating subsequent phosphorylation on Ser/Thr residues of DCC. One can also hypothesize that the activity of Src family tyrosine kinases is required as an initiation event to activate the Ser/Thr kinase(s), leading to full phosphorylation of the receptor DCC. In the present paper, we have found that tyrosine phosphorylation of DCC is highly increased in the presence of Fyn-CA, but not Src-CA, when both proteins are expressed in N1E-115 cells. Also, the basal level of tyrosine phosphorylation of DCC is inhibited in the presence of PP2 or SU6656, as well. In addition, we have demonstrated that Fyn, but not Src, phosphorylates the intracellular domain of DCC in vitro and that DCC tyrosine phosphorylation is completely impaired in $Fyn^{-/-}$ CN. Therefore, we propose that Fyn is the major kinase involved in the regulation of DCC phosphorylation.

We identified Y1418 as the major site of phosphorylation by Fyn tyrosine kinase in vitro; however, when each of the DCC tyrosine mutant proteins is coexpressed with Fyn in N1E-115 cells, tyrosine phosphorylation of both Y1261F- and Y1418F-DCC mutants shows a twofold decrease compared with that of DCC. These findings suggest that both Y1261 and Y1418 are phosphorylation target sites of Fyn in vivo. Interestingly, only mutation of Y1418 impairs the ability of DCC to induce neurite outgrowth in N1E-115 cells, similar to inhibition of neurite outgrowth by PP2 and SU6656. Therefore, our data suggest that more than one tyrosine of the cytoplasmic tail of DCC is phosphorylated in vivo by Fyn, but only phosphorylation of DCC Y1418 by Fyn is required to mediate neurite outgrowth in N1E-115 cells. Consistent with our data, more than one tyrosine residue in the cytodomain of *C. elegans* UNC-5 is phosphorylated in vivo, but only Y482 in the juxtamembrane region is critical for UNC-5 axon guidance function in vivo (Killeen et al., 2002). Y1418 in the cytoplasmic tail of DCC is well conserved among the vertebrate species and *X. laevis*, whereas it is not conserved in *C. elegans* UNC-40 and *D. melanogaster* Frazzled. However, there are other tyrosine residues that may serve a similar function to Y1418 in UNC-40 and Frazzled. Interestingly, Y1418 is also conserved within the cytoplasmic tail of neogenin, a member of the DCC family of proteins. Netrin-1–neogenin interaction has been shown recently to play a role in the morphogenesis of mammary glands, which are nonneural tissues (Srinivasan et al., 2003). It will be of interest to address the question of whether the conserved tyrosine in neogenin is phosphorylated and plays a similar role in cell migration and nonneural organogenesis.

We and others have shown previously that DCC induces the activation of the small GTPase Rac1 in response to Netrin-1 stimulation (Li et al., 2002b; Shekarabi and Kennedy, 2002). Rac1 activity is required for DCC to induce neurite outgrowth in N1E-115 neuroblastoma cells; however, the cascade of molecular events from DCC that leads to Rac1 activation is still poorly understood. Here, we show that phosphorylation of Y1418 in DCC is required to trigger Rac1-GTP loading in a Netrin-1–dependent manner. Interestingly, mutation of the other tyrosine residues in the intracellular domain of DCC did not interfere with Rac1 activation. Consistent with the data obtained in N1E-115 cells, these results emphasize the essential role that Rac1 plays in Netrin-1–mediated neurite outgrowth.

Based on these data, we propose the following model (Fig. 9): Netrin-1 binding to DCC induces rapid phosphorylation of Y1418 in the vicinity of the P3 region in the intracellular domain of DCC by Fyn. This primary event, in turn, might initiate directly or indirectly the activation of a specific unknown guanine nucleotide exchange factor that leads to the GTP loading of Rac1. Activation of Rac1 results in actin assembly at the plasma membrane that leads to axon outgrowth and attraction. The adaptor molecule Nck-1 is constitutively bound to DCC, and its role in the regulation of phosphorylation of DCC remains elusive. However, it is possible that Nck-1 serves to bring Fyn in proximity to DCC via its free SH2 domain, in response to Netrin-1.

Recent data have shown that signaling through calcineurin and nuclear factor for activated T cells (NFAT) proteins is implicated in the regulation of embryonic axon outgrowth in response to neurotrophins and Netrins. It has been proposed that Netrin-1 induces a calcineurin/NFAT molecular cascade controlling the axon outgrowth function of DCC but not the growth cone attraction (Graef et al., 2003). Here, we suggest that phosphorylation of DCC and the subsequent intracellular events leading to Rac1 activation are involved in axon outgrowth and in Netrin-1–mediated axon attraction. It will be of great interest to determine, in future studies, which proteins interact with the specific phosphorylation sites of DCC, and how they affect the outgrowth and chemotropic responses of axons to Netrin-1.

Materials and methods

DNA constructs and site-directed mutagenesis
The tyrosine residues located at positions 1261, 1272, 1361, and 1418 of the intracellular domain of full-length DCC in pRK5 vector (Li et al., 2002b) were substituted with phenylalanine residues using a PCR oligonucleotide-directed mutagenesis, according to standard protocols. The following primers were used: Y1261F (5′-GAAAGTGCCCAGTTCCCAGGAATCCTCCCG-3′); Y1272F (5′-CCCACATGTGGATTTCCGCATCCACAGTTC-3′); Y1361F (5′-CAAAAGTCCTTTTACACCGCTTTTGTCG-3′); and Y1418F (5′-GCCGATGTATTCGAACAGGATGACTTG-3′). PCR fragments were inserted, with HindIII, into pRK5-DCC to create the mutants. The intracellular domains of DCC mutants (DCC-C) were subcloned into the pGEX vector to produce GST fusion proteins (Li et al., 2002b). Fyn, Fyn-Y531F, and Fyn-K299A constructs were provided by S. Stamm (University of Erlangen-Nuremberg, Erlangen, Germany), and Src-Y538F and Src-K298R constructs were a gift from S.A. Laporte (McGill University, Montreal, Quebec, Canada).

Ligand and drug treatments
Recombinant Netrin-1 was produced and purified as described previously (Serafini et al., 1994). Netrin-1 was used at a final concentration of 500 ng/ml to stimulate dissociated CN and COS-7 cells and at 160 ng/ml to treat spinal cord explants. Genistein (Sigma-Aldrich) and PP2 (Calbiochem) were used at final concentrations of 100 and 10 μM, respectively. SU6656 (Calbiochem) was used at final concentrations of 0.5, 1, and 2 μM.

Cell culture
E13 rat or E11.5 mouse CN were dissected from dorsal spinal cords as described previously (Tessier-Lavigne et al., 1988; Shekarabi and Kennedy, 2002). Isolated CN and N1E-115 were plated on 60-mm dishes or on coverslips coated with 20 μg/ml of laminin-1 (Canlab) and grown as described previously (Li et al., 2002b).

Cell transfection
We transfected N1E-115 cells using the LipofectAMINE reagent (Invitrogen), according to the manufacturer's protocol, with 1 μg each of the fol-

lowing constructs: pRK5 empty vector, DCC, DCC-Y1261F, DCC-Y1272F, DCC-Y1361F, DCC-Y1418F, Fyn, Fyn-K299A (Fyn-DN), Fyn-Y531F (Fyn-CA), Src-Y538F (Src-CA), and Src-K298R (Src-DN). We cotransfected COS-7 cells with 4 µg each of pRK5 empty vector, DCC, DCC-Y1261F, or DCC-Y1418F, together with 1 µg of pRK5-Myc-Rac1, using the DEAE-dextran method as described previously (Olson et al., 1995). 24 h later, the cells were serum-starved overnight before a 5-min treatment with Netrin-1.

Immunofluorescence

Transfected N1E-115 cells and E13 rat CN plated on laminin-1–coated coverslips were fixed for 10 min in 3.7% formaldehyde/PBS. The cells were permeabilized for 5 min in 0.25% Triton X-100 in PBS and incubated for 30 min in 0.1% BSA. Next, they were incubated with anti-DCC antibodies (G97-449; BD Biosciences) and rhodamine-conjugated phalloidin (Sigma-Aldrich), and then with FITC-coupled secondary antibodies (Sigma-Aldrich). The coverslips were mounted on Mowiol (Calbiochem) and the cells were examined with a microscope (Axiovert 135; Carl Zeiss MicroImaging, Inc.) using an oil immersion 63× planapochromat lens (Carl Zeiss MicroImaging, Inc.). Images were recorded with a digital camera (DVC) and analyzed with Northern Eclipse software (Empix Imaging Inc.).

Immunoprecipitation

Transfected N1E-115 cells or E13 rat or E11.5 mouse CN treated with Netrin-1 for different periods of time were lysed in 20 mM Hepes, pH 7.5, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, and 10 µg/ml aprotinin and leupeptin. The supernatants were precleared for 2 h with protein G–Sepharose beads before being incubated overnight at 4°C with 20 µl of protein G–Sepharose beads and 2.5 µg anti-DCC antibodies. Protein samples were resolved by SDS-PAGE and transferred on nitrocellulose. The membranes were immunoblotted with the following antibodies: anti-pY (4G10; Upstate Biotechnology), anti-Fyn (provided by A. Veillette, Institut de Recherche Clinique de Montreal, Montreal, Canada), and anti-DCC.

Explant assays

E13 rat and E11.5 mouse dorsal spinal cords were dissected as described previously (Tessier-Lavigne et al., 1988). Dorsal explants embedded in three-dimensional collagen type I gels (BD Biosciences) were treated either with Netrin-1 alone or with Netrin-1 and either PP2 or SU6656. Images were captured after 36 h with a digital camera on a microscope (Axiovert 135) with a 40× phase-contrast objective lens. The total length of the axon bundles was calculated using Northern Eclipse software.

Fyn knockout mice

Male and female homozygous null breeding pairs for *Fyn*−/− mice (B6; 129S-Fyn^tm1sor) were purchased from the Jackson Laboratory. Genomic DNA was made from tail tips clipped at weaning, to genotype the mice using a PCR assay as described by the manufacturer (The Jackson Laboratory).

Retinal cultures and growth cone turning assays

Eye primordia were dissected from stage 24 *X. laevis* embryos and plated as explant tissue on coverslips coated with 50 µg/ml fibronectin (Roche). 24-h cultures were used in the growth cone turning assay as described previously (de la Torre et al., 1997). Growth cones that actively grew in a straight line for 30 min before the beginning of the experiment were chosen. The responses of these growth cones to an applied concentration gradient were recorded for 45 min using a CCD video camera (Cohu) and Scion Image capture software (shareware). Stable Netrin-1 gradients were formed by pulsatile ejection of a concentrated solution of 1 µg/µl Netrin-1 from a 0.5–1.0-µm–tip glass capillary pipette placed at a 45° angle from the actively extending growth cone. Control solutions consisted of 1% BSA in PBS. Upon completion of the experiment, only actively extending growth cones (>5 µm growth) were analyzed. Experiments were performed in a blinded fashion such that only after the analysis of the recorded video was the identity of the pipette solution revealed to the experimenter. The trajectories of the growth cones were traced onto a graph and the turning angles measured.

In vivo [^{32}P]orthophosphate labeling

E13 rat CN grown for 72 h on laminin-coated 60-mm dishes were starved in a phosphate- and serum-free medium 2 h before being radiolabeled. CN were then incubated for 2 h in the presence of 0.5 mCi/ml [^{32}P]orthophosphate. The cells were stimulated with purified Netrin-1 for 5, 10, and 30 min. The endogenous DCC was immunoprecipitated from the cell lysates, and proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The radiolabeled proteins were visualized by autoradiography at −80°C.

Phospho–amino acid analysis

The bands corresponding to phosphorylated DCC obtained after 5 or 30 min of Netrin-1 stimulations were subjected to a phospho–amino acid analysis as described previously (Boyle et al., 1991). Radiolabeled amino acids were detected by autoradiography at −80°C, and the signals obtained were quantified by densitometry.

Protein purification

Cultures of *Escherichia coli* strain DH5α transformed with pGEX-DCC-C, pGEX-DCC-C-Y1261F, pGEX-DCC-C-Y1272F, pGEX-DCC-C-Y1361F, or pGEX-DCC-C-Y1418F were subjected to protein production and purification as described previously (Li et al., 2002b).

In vitro kinase assay

20 µg of either GST or GST fusion proteins containing DCC-C or DCC-C-Y1261F, DCC-C-Y1272F, DCC-C-Y1361F, and DCC-C-Y1418F, as well as 5 µg myelin basic protein, were incubated for 10 min at 30°C with or without 20 ng/µl of active Fyn (Upstate Biotechnology) in buffer A (200 mM Tris-HCl, pH 7.5, 0.4 mM EGTA, and 0.4 mM sodium orthovanadate) in the presence of 10 µCi/µl γ-[^{32}P]ATP. 20 µg GST and GST-DCC-C as well as 5 µg of Src substrate peptide (KVEKIGEGTYGVVYK) were incubated for 10 min at 30°C with 10 ng/µl of Src active kinase (Upstate Biotechnology) in buffer B (100 mM Tris, pH 7.2, 125 mM MgCl$_2$, 25 mM MnCl$_2$, 2 mM EGTA, and 0.25 mM sodium orthovanadate) in the presence of 10 µCi/µl γ-[^{32}P]ATP. The phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography at −80°C. A range of DCC-C concentrations, from 25 to 100 nM, were used to estimate the Km value using the Lineweaver-Burk equation.

Rac1 activation assay

The amounts of GTP-loaded Rac1 in COS-7 cells expressing or not expressing DCC, DCC-Y1261F, DCC-Y1272F, DCC-Y1361F, or DCC-Y1418F mutant proteins, together with Myc-Rac1 and treated for 5 min with Netrin-1, were measured using a pull-down assay as described previously (Li et al., 2002b).

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